Central Neuropathology and Clinicopathological Correlates in Equine Grass Sickness

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ABSTRACT

Equine Grass Sickness has traditionally been known as a dysautonomia, principally affecting parasympathetic neurons in the enteric nervous system. Studies of central neuropathology have been cursory and conflicting, examining different and occasionally poorly defined central structures in variable numbers of cases and control animals. There was no agreement on the association or severity of clinical signs with the severity of central pathological changes.

This study accurately describes the distribution of pathology in the brain of EGS cases. Chromatolytic neurons have a highly specific distribution which is unlike that reported in any other equine or human disease, but is apparently the same as in cats, dogs and hares with primary dysautonomias. The involvement of somatic efferent lower motor neurons suggests that EGS may be more correctly classified as a multisystem disease. This is a further incentive to search for a common aetiologic agent and may decrease the number of candidates under consideration.

The nature of the pathological insult to the central neurons remains undetermined but, unlike peripheral neurons, central neurons do not appear to be dying; this study was unable to demonstrate evidence of neuronal apoptosis, axonal pathology or muscle fibre type grouping in muscles innervated by chromatolytic neurons. Phosphorylated neurofilament epitopes were labelled in the soma of somatic and visceral lower motor neurons indicating an axonal transport problem, but no consistent expression of the cell stress protein ubiquitin was evident. Smaller, CGRP-expressing dorsal root ganglia neurons are more likely to be chromatolytic than large neurons and may contribute to the observed rhinitis sicca. Electron microscopy revealed classical chromatolytic changes and no inclusion bodies.

Electrodiagnostic examination of the blink reflex did not reveal a functional deficit of the facial nerve, and the characteristic ptosis of EGS cases was determined to be an expression of Horner's syndrome secondary to pathology to postganglionic sympathetic neurons. The response of the equine eyelid to alpha agonist eyedrops was defined and a significant difference found between control animals and EGS cases. The technique has been developed further as a useful non-invasive adjunct diagnostic test in Grass Sickness.

DECLARATION

I declare that I have composed this thesis and that all the work is my own.

Caroline Nora Hahn BS DVM MSc MRCVS

DEDICATION

To Nicolas,

the memory of whose suffering

will forever remind me why we study neurology.

ACKNOWLEDGEMENTS

I was under the delusion that my study was organised the day the Horserace Betting Levy Board generously offered to fund me, as there had been no mention in the 'How to do a Phd' instruction manual of how totally dependent a PhD project is on the help of friends, who should have known better, and acquaintances, who were never given a choice. Brian Summers, Bruce Currie, Jeanne Bell, Ian Griffiths, Neil MacIntyre and especially John Fazakerley all somehow managed to contain and instruct a graduate student claiming enthusiasm for the topic, but having no identifiable laboratory skills. I am particularly grateful for all the work done by Allison Beard, who cut over 1400 tissue sections and to Steve Mitchell, for processing the grids and being a competent and entertaining navigator on our electron microscopy spelunking excursions.

My PhD program unwittingly gave me a second chance to study at my *alma mater,* and I was very fortunate to be strongly influenced by Alexander deLahunta, my 'grandsupervisor', who spent many hours teaching me neuroanatomy in the classroom, over a microscope and via e-mail at highly unusual hours of the 'day', somehow managing to turn a subject I passionately disliked into one I have now have a mission to teach. A big thank you also to Louise Taylor who took time out of her difficult post-doc to teach the use of general linear models to someone who is best described as 'mathematically challenged'. It goes without saying that I continue to be indebted to my exceptional parents, who showed an enormous sense of humour by supporting me faithfully despite never quite understanding the absolute necessity of acquiring a *second* doctorate while working stupid hours, ignoring the concept of grandchildren and making less money than the housekeeper.

To all of you thank you.

There is no question however that in reality the completion of this thesis is entirely due

to the efforts of Professor Joe Mayhew. The concept of doing a PhD was firmly at the very top of my list of things never, ever to do but bizarrely became something that might be feasible, if not immediately recognisably desirable, the moment Joe suggested I become his graduate student. That was several lifetimes ago, and without doubt I would never have achieved this had he not then spent several years working hard at filling conflicting roles as teacher, taskmaster, therapist, mentor and particularly friend.

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Hahn CN, Mayhew IG and de Lahunta A. Neuropathology of the brain in equine grass sickness. Acta Neuropathologica. *In press.*

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List of Abbreviations

AGS	Acute grass sickness
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
ATPase	Adenosine triphosphatase
CCG	Cranial cervical ganglion
CGRP	Calcitonin Gene Related Peptide
CGS	Chronic grass sickness
CN	Cranial nerve
CVM	Cervical vertebral malformation
EGS	Equine grass sickness
EMND	Equine motor neuron disease
GSA	General somatic afferent
GSE	General somatic efferent
GVE	General visceral efferent
ILH	Intermediolateral horn of the spinal cord
LAOM	Levator anguli oculi medialis
LMN	Lower motor neuron
NRS	Non-immune rabbit serum
PBS	Phosphate buffered saline
SGS	Subacute grass sickness
TG	Trigeminal ganglion

Chapter One

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Equine Grass Sickness (EGS) is a disease of horses kept at grass with lesions of neuronal chromatolysis and loss principally in postganglionic sympathetic and parasympathetic neurons (Obel, 1955; Chandler and Brownlee, 1967; Barlow, 1969; Gilmour, 1975; Doxey *et al*, 1992; Pogson *et al*, 1992). Classical clinical signs include dysphagia, gut stasis, tachycardia, inappropriate sweating, trembling of axial and appendicular muscles, rhinitis sicca and ptosis. There are no non-invasive, simple diagnostic tests, clinical diagnosis being based on assessing the combination of demographic data, history and clinical signs.

EGS is prevalent in the UK, in restricted Northern areas of continental Europe and in Patagonia, South America (Uzal *et al*, 1991), and has not been reported in Australasia (Wood *et al*, 1997). Clinical cases of EGS in North America (P. Johnston, U. of Missouri, personal communication) and Finland (Riitta-Mari Tulamo, University of Helsinki, personal communication) were recently confirmed by histopathology (C.N. Hahn, unpublished observations).

The disease is known as a dysautonomia (Doxey *et al*, 1991b) but clinical signs of EGS do not fit clearly into an uncomplicated picture of autonomic disease. Cases usually do not have alterations in pupillary diameter, enophthalmos, corneal ulceration due to decreased tear production or overt orthostatic hypotension. There is no good human model of the disease although some histopathologic and clinical signs are shared with acute autonomic neuropathy (Hart and Kanter, 1990), idiopathic orthostatic hypotension (Roessmann *et al*, 1971) and particularly familial dysautonomia (Pearson *et al*, 1971; Tonholo-Silva *et al*, 1994).

Despite the recognition of EGS since the early part of this century and over 100 associated publications, the aetiology of EGS remains an enigma. Neither the restricted geographical and age distribution, nor the occurrence of primary dysautonomias with

markedly similar enteric and sympathetic ganglionic lesions in cats (Pollin and Griffiths, 1992), dogs (Schultze *et al*, 1997) and hares (Whitwell, 1991) have helped elucidate the pathogenesis or aetiology of this disease, nor has the correlation of pathological findings with the non-enteric clinical signs, specifically muscle trembling, ptosis and rhinitis sicca, been addressed in detail. The basis of this project came from discussions undertaken in 1995 at the first international workshop on equine neurodegenerative diseases (Hahn *et al*, 1997). The editorial prologue and epilogue are attached in appendix 1 as an introduction to the topic.

2. LITERATURE REVIEW

a) Neuroanatomy of the general visceral efferent system

A basic understanding of the anatomy of the autonomic nervous systems ([Gr] auto - self, nomos - law) is fundamental to a discussion of EGS and a brief review is warranted.

There are a number of semantic controversies (Anthoney, 1993) in defining the anatomy of the autonomic nervous system (ANS). Specifically, there is no consensus as to whether it consists of strictly a lower motor neuron (LMN) visceral efferent system (Peele, 1977), a motor system with central control (Jenkins, 1978) or whether it also includes afferent pathways in addition to central regulatory centres (de Lahunta, 1983). In addition, there is no agreement on whether the enteric nervous system is part of the ANS, consisting of parasympathetic postganglionic neurons, or whether it is a part of the ANS but separate from the sympathetic or parasympathetic system. The following description is limited to efferent and central components.

The ANS is a physiologic and anatomic system that includes the only motor system innervating those structures whose functions are primarily outside voluntary control. It

is divided into sympathetic (thoracolumbar) and parasympathetic (craniosacral) divisions on the basis of physiologic, anatomic and pharmacologic differences of component neurons. The general visceral efferent (GVE) system is the lower motor neuron portion of the ANS and unlike the general somatic efferent (GSE) system consist of two neurons, pre and postganglionic, between the central nervous system (CNS) and the organ to be innervated. See Table 1-1 for a summary of the classification of functional systems.

Integrative centres are situated in the midbrain, pons, medulla and particularly the hypothalamus. The rostral and caudal portions of the hypothalamus are associated with the parasympathetic and sympathetic systems respectively and receive afferent input from the cerebrum, thalamus and general visceral afferent system. In turn the hypothalamus influences metabolic centres in the brainstem which affect GVE neurons in cranial and spinal nerves.

The sympathetic nervous system ([Gr] sym - with, pathos - emotion) has preganglionic cell bodies situated in the intermediolateral grey column in approximately the first thoracic to fifth lumbar spinal cord segment. Axons leave the cord via a spinal nerve and white rami communicantes to join the paravertebral ganglionated sympathetic chain which runs from the first cervical to the caudal vertebrae. The sympathetic trunk contains ganglia of postganglionic neurons and is separated into three distinct ganglia cranially, the cranial cervical, middle cervical and cervicothoracic ganglia, and more diffuse ganglia associated with the thoracic, lumbar and sacral vertebrae. In the horse the middle cervical and cervicothoracic ganglia are closely apposed and traditionally referred to as the stellate ganglion. It is unclear whether authors always include the middle cervical ganglion when discussing pathology to the stellate ganglion so the word 'stellate' is still used unqualified throughout this review.

On entering the sympathetic trunk, the route of the preganglionic neuron varies. Some synapse with a multipolar post ganglionic neuron in a paravertebral ganglion, either

with the first one they encounter or, by turning within the trunk, with a more cranial or caudal ganglion. The postganglionic axon exits the trunk via a grey ramus communicans and spinal nerve to innervate smooth and cardiac muscles and glands. Other preganglionic neurons leave the sympathetic chain directly via thoracic or splanchnic nerves to synapse in a prevertebral ganglion, principally the coeliacomesenteric and caudal mesenteric ganglia. Parasympathetic axons pass through the prevertebral ganglia but do not synapse there. The paravertebral ganglia principally contain vasomotor, sudomotor and pilomotor neurons while prevertebral ganglia innervate the viscera of the abdomen. Neurons in the paravertebral ganglia of the thoracic and cervical regions however serve a dual function due to the absence of prevertebral ganglia in those regions. The prevertebral ganglia are connected by a myriad of nerve fibres forming plexuses. Caudal to the caudal mesenteric ganglion, sympathetic postsynaptic axons make up the hypogastric nerve to anastomose with rami of the parasympathetic pelvic nerves as well as sympathetic postganglionic axons from sacral paravertebral ganglia to form a dense pelvic plexus.

The activity of the parasympathetic nervous system ([Gr]: para - next to) is much more restricted than the sympathetic nervous system as preganglionic fibres terminate mostly in ganglia associated with the organ innervated. Preganglionic parasympathetic neurons are also located in the neuraxis, separated into cranial and caudal divisions.

In the cranial division, preganglionic parasympathetic neurons in the medulla oblongata are associated with cranial nerves (CN) III, VII, IX, X and XI in a nuclear column, the GSE column, dorsolateral to nuclei containing neurons innervating skeletal muscles and ventral to the fourth ventricle (see Figure 1-1). The ganglia containing postganglionic neurons innervated by preganglionic axons in cranial nerves III, VII, IX, and XI are collectively called cephalic ganglia, whereas preganglionic vagal axons synapse in cell bodies in the wall of the viscera innervated. Locations of the pre and postganglionic cranial outflow components and the structures innervated are outlined in Table 1-2.

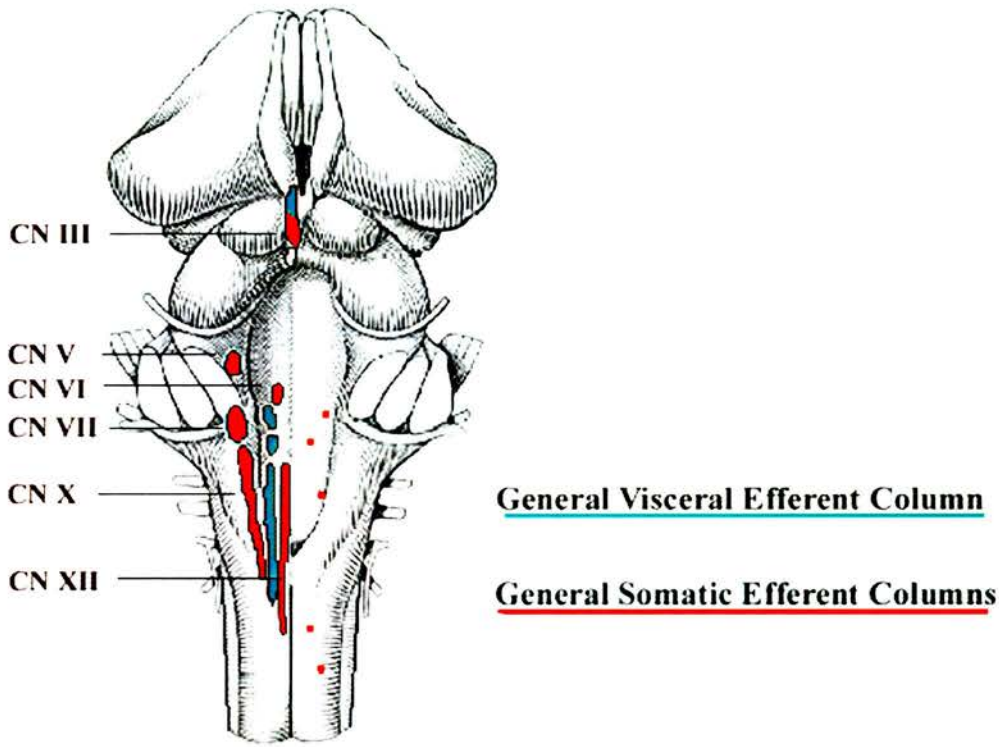
In the caudal division, preganglionic neurons are found in the lateral intermediate grey columns of sacral segments of the spinal cord. Axons exit the cord with the ventral roots at these levels to join the spinal nerves, and then separate as ventral branches that unite to form a pelvic nerve on each side. Axons in the pelvic nerve synapse with postganglionic neurons in the pelvic or hypogastric plexuses, or terminal ganglia within walls of thoracic and abdominal viscera. The majority of neurons within the pelvic plexus are thought to be parasympathetic.

Table 1-1: Functional classification of the nervous system

Functional System	Components
General Somatic Afferent (GSA)	Pain, temperature, touch. (spinal nerves, CN V)
Special Somatic Afferent	Vision (CN II), hearing (CN VIII)
General Visceral Afferent	Organ content and displacement, chemical changes. (Spinal nerves, CN VII, IX, X)
Special Visceral Afferent	Taste (CN VII, IX, X), Smell (CN I)
General Proprioception	Muscle and joint movements (spinal nerves, CN V)
Special Proprioception	Vestibular system, balance (CN VIII)
General Somatic Efferent (GSE)	Striated skeletal muscle (spinal nerves, CN III, V, VII, IX, X, XI)
General Visceral Efferent (GVE)	Smooth and cardiac muscle, glands. (sympathetic - spinal and splanchnic nerves, parasympathetic - CN III, VII, IX, X, XI)

From de Lahunta (1983, p310).

Figure 1-1: Functional organisation of cranial nerve nuclei in the brainstem.



Modified from de Lahunta (1983, p96).

Table 1-2: Parasympathetic general visceral efferent structures

Preganglionic cell body	Cranial Nerve	Postganglionic cell body	Postganglionic axon	Organs innervated
Edinger-Westphal nucleus	III	Ciliary ganglion	Short ciliary nerves	Sphincter of pupil ciliary muscle
Parasympathetic nucleus of VII	VII	Pterygopalatine, mandibular and sublingual ganglia	CN V maxillary CN V mandibular	Lacrimal, palatine and nasal glands, and mandibular and sublingual salivary glands
Parasympathetic glossopharyngeal nucleus	IX	Otic ganglion	CN V mandibular	Zygomatic and parotid salivary glands
Parasympathetic nucleus of X	X	Postganglionic neurons in wall of viscera		Cardiac muscle, smooth muscle and glands of respiratory and digestive system

Modified from Jenkins (1978, p310).

b) Clinical categories of equine grass sickness

Historically the classification of cases into the three categories, acute (AGS), subacute (SGS) and chronic (CGS) grass sickness, were defined strictly by duration of disease (Gilmour, 1988; Doxey *et al*, 1991b). Since this classification relies on the somewhat variable level of owner observation, a classification by clinical severity was used for purposes of this study.

AGS cases are defined as animals with severely decreased gut motility leading to small intestinal distension and gastric reflux in addition to the classical signs of EGS, and horses are invariably euthanised on humane grounds within 48 hours of first showing clinical signs. Subacute cases have somewhat milder clinical signs and a characteristic 'tucked up' stance, do not present with distended small intestines but have colonic

impactions. These are usually euthanised within one week of showing clinical signs. Chronic cases are defined as horses with EGS but without profound changes on rectal examination and milder dysphagia and ileus. Evidence of rhinitis sicca is very common in this group and a small proportion of the chronic cases recover with intensive nursing care.

c) Equine grass sickness neuropathology

The first comprehensive histopathological examination of a case of EGS was published by Holman *et al* (1944), reviewing cases sent to the Moredun Research Institute over the previous 20 years. A detailed examination of the nervous system was carried out, including midbrain, pons and medulla, vagus, splanchnic and phrenic nerves, spinal cord and coeliacomesenteric, thoracic, jugular (proximal GSA CN X) and spinal ganglia. No signs of pathology were found in the central nervous system (CNS) or peripheral nerves, however nor were significant lesions noted in any of the autonomic ganglia.

A detailed histopathological description of grass sickness was reported by Obel in 1955. The sympathetic trunk, prevertebral ganglia, vagal-glossopharyngeal nucleus in the medulla oblongata, the nodose plexus and the spinal cord were examined. The most marked changes were found in the cranial cervical ganglia and the stellate ganglia, while caudal mesenteric ganglia were often affected to a lesser degree. In the more severe, acute cases, swollen, chromatolytic neurons in sympathetic ganglia with eccentrically placed enlarged nuclei and nucleoli were noted in sympathetic ganglia. Chromatolytic and vacuolated neurons lacking nuclei were also sometimes seen in spinal cord lateral and ventral horns. Some soma contained small vacuoles that progressed to making the cell appear enlarged and spongy, either peripherally, centrally or in alternating zones. Karyopyknosis or karyolysis was described in the same cells. There appeared to be no reaction by surrounding satellite cells but these were noted to have proliferated into nodules of Nageotte where neurons had entirely disappeared.

Enlarged axons and eosinophilic granules resembling inclusion bodies were observed in some cells but inflammation was conspicuously absent. In less severe, chronic, cases damage to neurons was thought to be as extensive as in acute cases but sympathetic ganglia were greatly depleted. Extensively vacuolated, chromatolytic 'cell shadows' and shrunken neurons with pyknotic nuclei were described in ganglia. The histopathological similarity to changes associated with poliomyelitis, that unlike grass sickness results in inflammation, and scrapie, that does not, was considered. Clinical signs of EGS were attributed to increased sympathetic activity.

In 1959 Brownlee and Mahaffey described degenerative changes of neurons in the coeliacomesenteric ganglia characterised by chromatolysis, the presence of small and occasionally large vacuoles and nuclear pyknosis in acute and subacute cases of grass sickness as well as in 3 out of 15 control horses. Brownlee (1965), remarked on the relative paucity of neuronophagia of degenerating neurons. Howell *et al* (1974) noted the numerous degenerate, foamy and necrotic neurones in the coeliacomesenteric ganglia of cases of grass sickness which were not present in horses that died from other causes. Vacuoles, pyknotic nuclei and some lymphocytes and macrophages were also observed. Very similar changes have been described in the coeliacomesenteric ganglia of cases of mal seco (Uzal *et al*, 1992).

A number of other publications have addressed the central histopathological changes found in EGS and in mal seco. Barlow (1969) examined a limited number of brain nuclei in two acute, three chronic and one case that had clinical sings of acute grass sickness but no pathological changes in any of the tissues examined. The facial, lateral vestibular, hypoglossal and dorsal motor vagal nuclei were normal in the acute cases but showed some pathological changes in the chronic cases. Similarly, the oculomotor nucleus was affected in the three chronic cases but not in one of the acute cases. No pathology was noted in dorsal root ganglia, but abnormal nuclei in the spinal cord ventral horns were seen in two out of three acute cases. Lipofuscin granules were present in normal and abnormal neurons and neuronophagia was not observed. It was

concluded that the CNS changes may be a specific part of the pathology of grass sickness with the extent depending on the duration of clinical signs.

A more extensive survey, looking at 35 cases of EGS, was performed by Gilmour (1973b) who concluded that lesions were more extensive and severe in acute and subacute cases than in chronic cases. Specifically, pathological changes were seen in all cases in the stellate, coeliacomesenteric, ciliary and dorsal root ganglia, the thoracic sympathetic chain, oculomotor nuclei and intermediolateral horns of the spinal cord. 26 of 32 cases had abnormalities noted in CN VII and other nuclei, including the nuclei of CN V, VI, VIII, GVE X, XII and the accessory cuneate nucleus were noted to be affected in some cases but this was not quantified. The very extensive changes found in autonomic neurons present in acute cases was felt to indicate that damage to extra-enteric tissue precedes, or occurs coincidentally with, alimentary dysfunction.

Wright and Hodson (1988) on the other hand could not correlate the extent of central changes with the clinical severity i.e. an acute versus chronic clinical course. Ten EGS cases (2 AGS, 5 SGS, 34 CGS) and one laminitic horse were examined. A number of nuclei and structures were specified as containing lesion. These include the nuclei of CN III, VI, VII, VIII, GVE X, XII as well as the spinal trigeminal and primary sensory nucleus of V, red nucleus and a number of structures and in the prosencephalon and brainstem. The neuroanatomical accuracy of this study might be questioned however as neurons were noted in the medial longitudinal fasciculus and the 'fields of forel', both of which are white matter tracts. Few structures were examined in all the horses. Lesions were noted to consist of a loss of cytoplasmic staining within the cytoplasm resulting in a homogenous ground glass appearance. Pathology in the reticular formation was most consistent, changes being present in seven of the eleven horses. In only two horses were abnormal neurons found in the ventral horn of the spinal cord, however the spinal cord segment and number of spinal cord sections examined was not detailed. Two of eleven nuclei examined in the laminitis case also contained abnormal neurons. The authors concluded that given the diversity of the nuclei involved and since

only two to three neurons per nucleus were affected, CNS involvement was likely to be non-specific.

This supposition was supported by Hodson *et al* (1984b) who, unlike Gilmour (1973b) argued that cytopathological changes in non enteric neurons appear to be similar to retrograde degenerative lesions after axotomy and could therefore be a secondary phenomenon following alimentary damage. This would lend weight to the concept that the CNS changes are non specific (Wright and Hodson, 1988). No effort was made to establish whether chromatolytic central neurons, be they somatic or visceral, were pathologically affected in the same manner as peripheral visceral neurons and go on to die. Secondary axonal or muscle pathology of structures innervated by cranial nerves have not been investigated.

In a histopathological study of the brain stem nuclei of 3 horses with mal seco, changes were found that consisted of chromatolysis, cytoplasmic vacuoles, eosinophilic spheroids, and pyknotic and eccentric nuclei. These changes were most severe in the nuclei of CN III, VI and VIII (Uzal *et al*, 1994). It was suggested that the results provided further evidence that mal seco and grass sickness may be the same disease.

Gilmour (1975) undertook a detailed light and ultrastructural examination of the stellate ganglion. Pyknosis and cytoplasmic vacuolation, possibly originating from granular endoplasmic reticulum was prominent, along with increased microtubules with an absence of normal endoplasmic reticulum. Mitochondrial structure was similar to that found in control samples. Numerous 5-15 μm, round, argyrophilic, eosinophilic bodies surrounded by satellite cell processes were seen by light and electron microscopy in perineuronal supporting tissue and in recesses in perikarya of normal and degenerate neurons. These neurons contained mitochondria and vacuoles in vesiculotubular material and were surrounded by a single limiting membrane and a sheath of satellite cell processes. They were thought to represent dystrophic axons as similar changes had been described in experimental and infantile neuraxonal dystrophy (Cowen and

Olmstead, 1963). Hodson *et al* (1987) and Hodson and Wright (1984b) described three types of neurons ultrastructurally in the coeliacomesenteric ganglia of 15 EGS cases. The most common were chromatolytic neurons with marginated Nissl substance, crenated and peripheral nuclei and dense bodies in cytoplasm. Many abnormal axons were observed which were enlarged and showed accumulations, indicative of axonal flow disruption. In addition there were neurons which appeared to be undergoing dissolution with prominent dilations of cisternae and a crenated nucleus, while another type was thought to be showing signs of recovery, containing aggregations of dense bodies and neuronal autophagic vacuoles. The overall pathology was felt to be compatible with retrograde lesions secondary to pathology of axons.

Primary morphological damage to axons was also suspected in work identifying accumulations of nor-adrenaline in nerve fibre tracts within the stellate ganglion of affected horses (Gilmour, 1976). These accumulations conformed with focal axonal fragmentation and formation of argyrophilic fibrillar tangles seen in silver-stained sections. This was supported by work showing that affected neurons have a marked increase in dopamine-beta-hydroxylase, an enzyme associated with noradrenaline synthesis, which may indicate a failure to be transported down the axon with resultant accumulation in the perikaryon (Griffiths *et al*, 1993).

Transmission studies in which blood or serum from grass sickness cases was administrated intraperitoneally to experimental ponies resulted in prevertebral ganglia pathology and unspecified brainstem nuclear pathology indicated that the suspected toxin was contained in a plasma protein fraction of molecular weight of 30,000 Da or greater (Gilmour and Mould, 1977). Its identity however has not been determined as toxicity appears to be inactivated during extraction procedures (Johnson, 1985) and could not be detected by polyacrylamide gel electrophoresis (Johnson *et al*, 1983) or thin layer chromatography (Pemberton *et al*, 1990).

In order to demonstrate that a causative agent of EGS may reach neurons by retrograde axonal transport, pooled sera from two acute cases of EGS were injected into the parotid salivary gland of ponies (Griffiths *et al*, 1994a). The parotid salivary gland receives its sympathetic innervation from the ipsilateral cranial cervical ganglion. Seven days later a few neurons in the cranial cervical ganglia were found to be chromatolytic in four out of five cases injected with acute serum. No clinical signs were apparent. Stellate and coeliacomesenteric ganglia, which would have a haematogenous connection, were unaffected however the otic ganglion, which supplies parasympathetic axons to the parotid gland, was not examined. No chromatolysis was noted in the one subject injected with serum from a chronic case, but a severe inflammatory infiltrate and neuronophagia was apparent which was speculated to be attributable to antibodies formed by the chronic cases. One further pony received serum fractionated to a molecular weight of above or below 30 KD into the left and right parotid glands. Chromatolysis was only observed in the ganglion on the side of the sample of the greater molecular weight. The paucity of numbers of neurons affected was attributed to the small percentage of cranial cervical axons which innervate the parotid salivary gland and it was concluded that retrograde axonal transport of the neurotoxin is likely. Rodents have been shown to retrogradely transport toxin at a rate of 180mm/day (Stockel *et al*, 1975), Thus an intestinal toxin could reach enteric neurons very quickly and theoretically reach all other ganglia within one to two days.

The search for potential aetiologic agents in grass sickness has been hampered by the lack of a consensus of the pathogenesis. A limited amount of work has addressed the associated cytopathology in EGS, and has focused on peripheral GVE neurons (reviewed further in chapter 3). Pathology was marked by a reduction in neuropeptide-containing ganglionic and mucosal endocrine cells (Sabate *et al*, 1983; Bishop *et al*, 1984) and changes to a wide array of cytoskeletal elements and an apparent absence of Golgi apparatus (Griffiths *et al*, 1993). The authors commented that a complete loss of the Golgi apparatus is very unusual in neuropathology but is not a known consequence

of axonal disease. Neurons from experimental ponies to whom grass sickness serum was administered by intraperitoneal injection (Gilmour, 1973a) had similar lectin - immunohistochemical profiles (IR Griffiths, unpublished observations).

d) Clinicopathological correlates in Equine Grass Sickness

Very little progress has been made correlating the clinical signs of EGS with the neuropathology since Greig (1928) postulated that the majority of clinical signs in EGS such as sweating, tachycardia and constipation were an expression of sympathicotonia.

The levels of plasma adrenocorticotropic hormone (ACTH), cortisol and catecholamines in cases of grass sickness and stressed and non stressed control horses (Hodson *et al*, 1984a; 1986) were assessed to try to support this hypothesis. Statistical evaluation determined differences in hormone levels between groups of seven horses with EGS (one acute, five subacute and one chronic), six horses with colic and 16 control horses before and after mild stress. Plasma levels of the hormones were higher in horses with acute and subacute grass sickness than in the other groups. It could not be concluded whether the hyperactivation of the sympathetic system was caused by stress as a result of the severity of the disease, or whether it also played a role in the aetiology. Unfortunately only one sample from a chronic case was analysed and pertinent clinical details, such as the presence of muscle fasciculation and heart rate, were not provided.

In an effort to further investigate eosphageal malfunction, that can be associated with dysphagia in EGS cases, the passage of a barium bolus from pharynx to stomach in 25 cases was monitored by image intensification radiography (Greet and Whitwell, 1987). A defect in oesophageal motility was detected in all 18 horses in which grass sickness was later confirmed at post-mortem. Chromatolysis in brainstem nuclei was noted but not detailed. Chromatolytic neurons were only occasionally observed in the oesophageal myenteric plexus and the severity of eosphageal dysfunction was not correlated with severity of neuronal pathology.

The only attempts made to associate clinical signs and pathology are related to the gastrointestinal tract. Scholles (1993) noted that the neuronal lesions of the small intestine may explain the intractable gastrointestinal propulsive deficit that characterises EGS, however severity assessment of the lesions was subjective. Quantitative work (Doxey *et al*, 1992; Pogson *et al*, 1992) has been carried out to establish whether a relationship exists between clinical severity and the extent of enteric neuronal pathology, damage to neurons in the small intestine and selected para and prevertebral ganglia. No connection between the clinical severity of acute or subacute dysautonomia and the amount of neuronal damage in the cranial cervical, stellate and coeliacomesenteric ganglia could be demonstrated. However a higher number of neurons and a lower proportion of damaged neurons were found in chronic cases. Similarly, in the jejunal submucosal and myenteric plexuses, chronic cases tended to have a greater number and percentage of healthy neurons. A related study focusing on the morphology of the enteric nervous system noted severe enteric neuropathy in all the cases with EGS but a widespread distribution in the acute cases versus more focal pathology in the distal intestine in chronic cases (Scholes *et al*, 1993).

Further work demonstrated that severe and extensive neuronal damage and loss occurred in the ileum irrespective of the severity of clinical signs (Doxey *et al*, 1995) and that the same pattern was seen in the small colon but it was always less obvious than in the jejunum. In no cases was enteric neuronal damage and loss seen without significant neuronal disruption also occurring in the coeliacomesenteric ganglia.

Conversely, attempts at experimental transmission by the intraperitoneal injection of serum from horses with acute grass sickness into four ponies resulted in prevertebral ganglionic pathology in the absence of enteric neuronal changes (Gilmour, 1973a). Spinal cord intermediolateral horn and brainstem nuclei were also affected however clinical signs did not develop in any of the cases. This was thought to indicate a neurotoxic factor in the blood of horses with acute grass sickness possibly of an insufficient dose required for the production of clinical disease.

A functional *in vitro* study to investigate isometric motility of equine intestine from horses with and without EGS was performed using strips of smooth muscle from the small intestine of EGS-affected and control horses taken immediately post mortem (Murray *et al*, 1994). Tissues obtained from horses suffering from acute grass sickness had the longest latency before a measurable response could be obtained and the ileum appeared to be damaged by EGS to a greater extent than the duodenum. By investigating the in-vitro response to bethanechol, a muscarinic receptor agonist, hypersensitivity was suggested in cases of acute grass sickness (Murray *et al*, 1994) while latency measurements following exposure to physostigmine suggested that the ileum of the EGS cases had a decreased number of active cholinergic neurons (Murray *et al*, 1997).

Despite the widespread central pathology associated with EGS noted in several papers (Barlow, 1969; Gilmour, 1973b; Wright and Hodson, 1988; Uzal *et al*, 1994), there are no clinical signs reported or discussed which could be correlated to the specific lesions, nor has correlating the non-enteric clinical signs such as dysphagia, trembling, rhinitis sicca and ptosis with specific pathologic lesions been attempted. Lesions in a number of structures might help explain some of the major clinical findings, but these have not been considered specifically and it is likely that functional systems other than the GVE system are involved in the aetiopathogenesis of some or all of these signs. These structures could include the basal nuclei, olfactory system, oesophagus, trigeminal ganglion and GVE lower motor neurons in the medulla that may be involved with dysphagia, as well as spinal cord LMNs, peripheral nerves and appendicular muscles that could be associated with trembling, and structures innervating the nasal mucosa that could explain the rhinitis sicca.

Knowledge of neuroanatomy and neuropathology is paramount for reliable interpretation of morbid entities involving the nervous system (Dexler, 1899) and therefore the close examination of forebrain, brainstem, spinal cord and peripheral

nervous system structures is required to establish the underlying pathology of these common and often severe clinical signs in EGS.

e) Dysautonomia in other species

Dysautonomia appeared in cats in the early 1980s (Key and Gaskell, 1982) as abruptly as it had done in horses over 70 years previously. These feline patients presented with signs consistent with autonomic failure including persistent bilateral pupillary dilation, dryness of the oral mucosa and reduced tear production, constipation, urinary incontinence, regurgitation, megaoesophagus and prolapse of the third eyelid (Sharp *et al*, 1984). Other aspects of neurological examination were reported to be normal but Griffiths (1982) noted slowed proprioceptive test responses in some cases. Chromatolytic neurons and neuronophagia were characteristically present in the autonomic ganglia and the enteric nervous system of the cats, as well as GSE LMNs in the ventral horn of the spinal cord and CN III, V, VII and XII, and GVE neurons in CN X (Griffiths *et al*, 1982; Pollin and Griffiths, 1987). Feline dysautonomia has been reported in a number of countries in northern Europe, as well as in New Zealand and the United States (Edney and Gaskell, 1988), countries in which EGS is not documented. The disease was not uncommon for several years but now appears to be confined to sporadic clusters (Symonds *et al*, 1993). Very similar clinical signs and pathological changes have been reported in a smaller number of dogs (Rochlitz and Bennett, 1983; Longshore *et al*, 1996).

Whitwell (1991) reported several cases of hares that had been found dead with gross PM changes reminiscent of grass sickness including marked cachexia, a distended stomach, colonic impaction and mucus in the rectal lumen. Leporine dysautonomia was confirmed in further animals (Griffiths and Whitwell, 1993; Whitwell, 1994) with peripheral histopathological and ultrastructural changes apparently identical to those in horses, cats and dogs. Similar lesions have been noted in wild rabbits (K.E. Whitwell, personal communication) and a recent report (Kik and van der Haage, 1999) recorded a

case of a llama (*Llama glama*) with clinical, gross and histopathological changes compatible with dysautonomia. The only lesions noted in the brain of that animal were chromatolytic neurons in the lateral cuneate nucleus.

f) Neurodegenerative diseases

The neurodegenerative disorders are slowly progressive diseases that lead to loss of specific neuronal populations and processes (Waggie *et al*, 1999) and are often classified as such on the basis of not fitting overtly into the categories of infectious, inflammatory, neoplastic or toxic disorders (Oliver *et al*, 1997). The use of this term appears to currently be out of favour with neuropathology text books (Graham and Lantos, 1997). Nevertheless the classification has merit in that it groups together some of the most devastating neurological diseases with unknown aetiologies. EGS may be included as a neurodegenerative disease (Gruys *et al*, 1994) on the basis of the death of defined groups of neurons, even though the disease can be rapidly progressive and does not invariably lead to the death of the animal.

A limited number of diseases of unknown aetiology leading to a loss of a defined group of neurons have been documented in horses. Premature early degeneration is exemplified by the cerebellar abiotrophies, known to be familial in Arabian, Gotland and Oldenberg breeds and present in Eriskay ponies (Hahn *et al*, 1999). Histologic abnormalities of the cerebellar cortex are principally a diffuse reduction of neurons in the granular and molecular layers, as well as degeneration and disorientation of Purkinje cells. A further disease with a familial component is equine degenerative myeloencephalopathy, characterised clinically by quadripedal ataxia and pathologically by prominent neuronal fibre degeneration in the spinal cord and brainstem and neuroaxonal dystrophy in the nucleus thoracicus and lateral cuneate nucleus (Mayhew *et al*, 1977). The exact pathogenesis of EDM is unknown however deficiency of vitamin E is a causative factor. Low dietary alpha tocopherol has similarly been shown to be of major importance in equine motor neuron disease (EMND), a disease resulting in

profound weakness and degeneration of alpha motor neurons in the spinal cord and specific brainstem nuclei (Cummings *et al*, 1990). It has a striking resemblance to progressive muscular atrophy, one of the forms of human motor neuron disease.

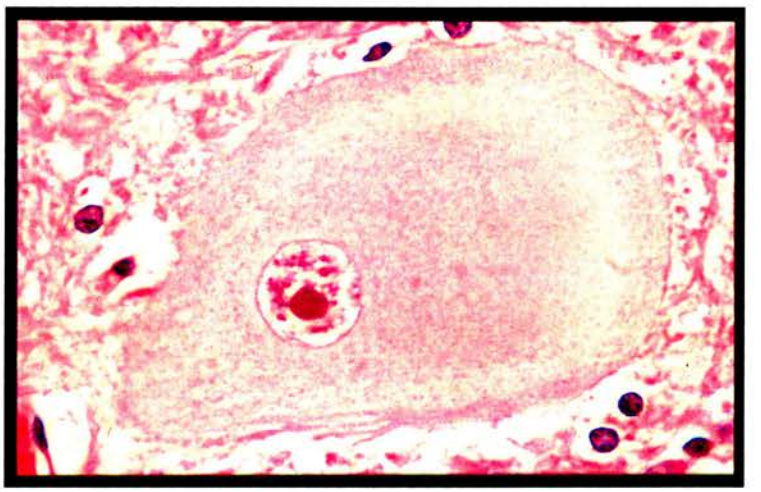
Objective

The objective of this work was to investigate the central nervous system pathology in cases of equine grass sickness, and to determine the neuropathological correlates of some of the characteristic clinical signs.

All the tissues used in this study were obtained from animals destined for euthanasia on clinical grounds.

Chapter Two

Central Histopathology



1. INTRODUCTION

The clinical and gross pathological picture of 'Grass disease' has been noted in the literature since 1927 , but it was Obel (1955), who published the first comprehensive review of the pathology of EGS. The morphology of neurons in the intestines and sympathetic ganglia were emphasised, but the appearance of 'ganglia cells' in the medulla oblongata and spinal cord was also referred to (Figure 2-1). These were described as being chromatolytic and containing vacuoles and eosinophilic granules resembling inclusion bodies.

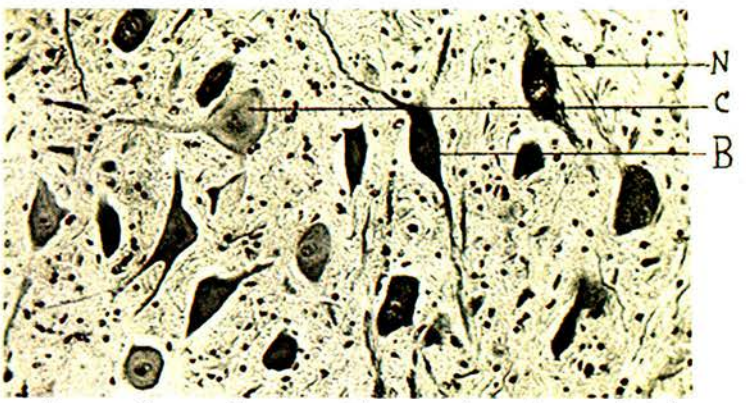
Four publications have addressed the central nervous system (CNS) pathology in grass sickness and mal seco (Barlow, 1969; Gilmour, 1973b; Wright and Hodson, 1988; Uzal *et al*, 1994), examining different, and occasionally poorly defined, central structures and variable numbers of cases and control animals. The central neuronal changes were described as being degenerative in character, involving initial central chromatolysis and eccentric, pyknotic nuclei. There was no agreement on the association or severity of clinical signs with the severity of central pathological changes. Barlow (1969) noted specific pathological changes correlated with disease duration, Gilmour (1973b) found more severe lesions in the more severe (acute) cases, and Wright (1988) concluded that the pathology was non-specific with no correlation to disease severity or duration. The latter paper also noted that in two instances abnormal neurons were also found in an orthopaedic control case. Gilmour (1973b) suggested that the very extensive changes found in peripheral autonomic neurons of acute cases indicated that damage to central tissue preceded, or occurred coincidentally with, alimentary dysfunction.

The disease is generally classified as a dysautonomia (Sharp, 1987) with some of the histopathological changes resembling human familial dysautonomia. The recognition of lower motor neuron (LMN) changes in the spinal cord of some cases however, has posed the question whether EGS could represent an overlap syndrome of equine motor

neuron disease (EMND) (Gerber and Gerber, 1997; Vandevelde, 1997), as has been proposed with some human neurodegenerative diseases (Hart and Kanter, 1990).

There has been no attempt to correlate any GSE central changes with somatic clinical signs. Muscle trembling is a prominent clinical sign of EGS (Pool, 1927; Holman *et al*, 1944) but its aetiology has not been addressed, nor has the correct descriptive terminology been resolved. Pathological fasciculations are commonly seen in human diseases of the lower motor neuron and are frequently found in patients with amyotrophic lateral sclerosis (Conradi *et al*, 1982). The presence of benign fasciculations and cramps in subclinical ALS patients implicates anterior horn cell dysfunction in the pathogenesis of muscle fasciculation-cramp syndromes (Fleet and Watson, 1986) and Sharp (1984) speculated that the muscle trembling see in EGS may be related to ventral horn cell involvement. It is thus relevant to determine if spinal cord LMN chromatolysis is correlated with trembling in EGS.

A thorough description of the central neuroanatomical distribution and severity of lesions in EGS is fundamental to further investigations into the pathogenesis of the cytopathology and the aetiology of clinical signs, and is the basis of this study.



The hypoglossal nucleus. The majority of the nerve cells are subject to chromatolysis (C) and a number of these are also subject to shrinking and basophilia (B). At N there is a normal ganglion cell.

Figure 2-1: From Obel, L. Studies on Grass Disease. J. Comp. Path. 1955.

2. MATERIALS AND METHODS

a) Case material

The brains of 72 horses (28 AGS, 15 SGS, 17 CGS cases and 12 non-neurological control animals) were obtained between 1995 and 1998 (Appendix 2). Animals were euthanised with a mixture of quinalbarbitone and cinchocaine (Somulose, Arnolds, UK). The head was disarticulated at the atlanto-occipital articulation and the brain was collected by sawing through the calvarium to harvest the brain as soon as possible after euthanasia, usually within 20 minutes.

For harvesting and processing the spinal cord tissue see section g below.

b) Tissue materials and methods

An incision was made at the level of the basal nuclei of the brain to allow fixative to enter the ventricles, and the brain and spinal cord were immersed in a large volume of 4% neutral buffered formaldehyde. After fixing for at least one week, the brain was blocked for processing. 4 mm blocks were taken at the level of the basal nuclei, hypothalamus, rostral colliculus, caudal colliculus and confluence of the peduncles, and the entire medulla oblongata was blocked to 1 cm caudal to the obex. Transverse blocks of tissue were labelled according to the nomenclature of Singer (1962) and embedded into paraffin blocks. The tissues were routinely processed and one 5 micron thick section was cut from the rostral surface of each block and processed to haematoxylin and eosin slides by the Moredun Research Institute. This one section from each block, was the only one where analysis was carried out, avoiding any problems with possible multiple counting of single neurons

c) Nuclear regions examined

Slides were examined blinded by the principal observer on a Nikon Optiphot-2 microscope. The entire section was surveyed for signs of pathology and special

attention was paid to the structures and nuclei in Table 2-2, present in most of the cases. Additional structures, such as central axons of cranial nerves and the parasympathetic nuclei of CN III, VII, and IX (rostral GVE column), were examined when available.

Each structure was identified and significant lesions were recorded. A pilot study indicated that chromatolysis of neurons (somata) in specific brainstem nuclei was a feature of EGS cases, and subsequently the proportion of chromatolytic neurons in affected nuclei was recorded in the majority of affected nuclei. For each nucleus in each section on which chromatolytic neurons were noted, the number of chromatolytic and normal cells examined was recorded. In a minority of sections (<20%) this proportion was estimated. The estimation of proportions was substantiated by counting a number of samples until the observer was confident that estimations were comparable to counted proportions. Neurons were only counted in fields in which there was evidence of chromatolysis. If the nucleus was bilateral, the combined data from both nuclei were included in the analysis, the validity of assuming symmetry of the proportions of chromatolytic neurons between nuclei on left and right sides of the brain having been determined (Wilcoxon Signed Rank Test, $p > 0.05$, Figure 2-2).

Neurons within a nucleus were counted using an eye-piece graticule (100 μm squares, Graticules, Pyser-SGI ltd) with neurons at the edges of the nucleus being included if the majority of the soma was within the grid. All neurons were included regardless of whether they contained a nucleus because of the concern that the number of chromatolytic neurons, with their characteristically swollen soma and eccentrically placed nucleus (Summers *et al*, 1995), would be underestimated if they were only included when a nucleus was present on the section. Also, a pilot study had determined that there was no significant difference in the proportion of chromatolytic neurons determined when counting neurons with or without nuclei (18 nuclei, Student's t-test, $p>0.95$). Data were entered into a customised database (Access, Microsoft Inc).

Summary statistics were calculated and displayed using statistical software (SigmanStat and SigmaPlot, SPSS Science).

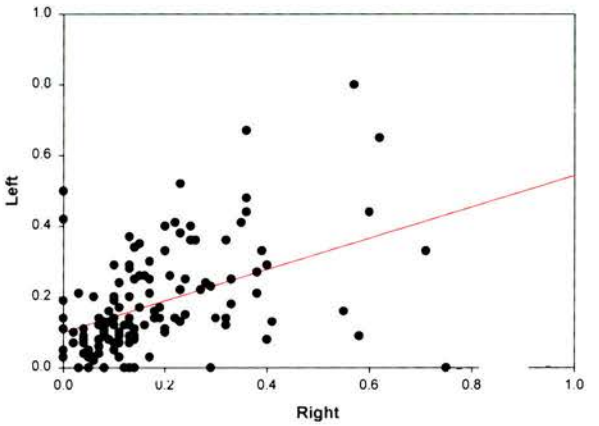


Figure 2-2: Proportion of neurons chromatolytic, left and right sides of brain.
Scatter plot of data with simple regression line from185 sections containing left and right nuclei. Regression line slope = 0.4.

d) Consistency of pathology

(i) Agreement of the presence of pathology between repeated sections through a nucleus

Due to the regular blocking method, individual nuclei were examined on multiple occasions depending on the length of the nucleus. Data from the nuclei of CN III, V, VII, XII or the parasympathetic nucleus of the vagus nerve (GVE X), were systematically examined to determine the proportion of cases in which an individual nucleus within a case was noted to contain chromatolytic neurons (somata) on at least one section, and to be unaffected on other sections.

(ii) Consistency of the proportion of chromatolytic neurons noted on repeated sections through a nucleus

To further evaluate repeatability, repeated sections of three nuclei were made and the percentage of chromatolytic neurons in the nuclei of GVE X and CN XII were evaluated in one case and the nucleus of CN VII was determined in another by counting the neurons in those nuclei on each section.

Data were analysed using a Kruskal-Wallis One Way Analysis of Variance on Ranks and the Kappa value of spatial repeatability was calculated.

(iii) Consistency of pathology within the general visceral efferent functional system

Similarly, the uniformity of pathology within a defined functional system (de Lahunta, 1983), the GVE column, was assessed by determining if the presence or absence of neuronal chromatolysis in the portion of the GVE column rostral to the nucleus of CN XII (GVE nuclei of CN III, VII and IX) was in agreement with pathology in the caudal portion (GVE X). Neurons in the nucleus of CN III were defined as being part of the GSE column if they were on the same section as neurons of the red nucleus and of a size similar to other somatic neurons. Small neurons in the sections further rostrally were defined as being part of the GVE column.

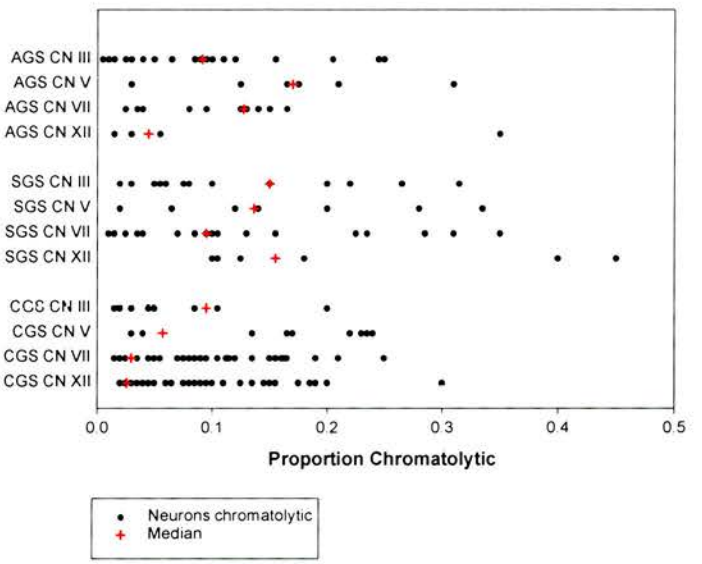
e) Severity of brainstem somatic lower motor neuron pathology

A pilot study determined that the somatic nuclei of CN III, V, VII and XII were the nuclei most consistently included with the strategic blocking method used in this study, and the severity of brain pathology was assessed by focusing on the these nuclei. Severity of pathology was assessed by determining the proportion of those nuclei affected in individual cases, the proportion of neurons chromatolytic in those nuclei and the proportion of sections containing these four nuclei examined in each case which

contained chromatolytic neurons. The proportion of cases with abnormalities noted in the nuclei of CN III, V, VII and XII in AGS, SGS and CGScases was determined and the 95% confidence intervals (CI) calculated from the Binomial distribution(Rohlf and Sokal, 1981).

Data of the proportion of chromatolytic nuclei of CN III, V, VII and XII, the proportion of chromatolytic neurons in those nuclei and the proportion of sections with chromatolytic neurons were initially examined using descriptive statistics of the raw data. Figure 2-3 shows an example of summary data. Pseudoreplication of data and the use of multiple data entries per animal precluded effective interpretation of the material.

Figure 2-3: Severity of chromatolysis, raw data



To avoid pseudoreplication, the total number of chromatolytic and normal neurons for a specific nucleus in an individual case was calculated by adding all the data from different sections of that nucleus. Since there was no evidence of neuronal death in nuclei in which chromatolysis was noted, the median total number of neurons counted in affected nuclei was used to estimate the number of neurons present on the small proportion of normal sections on which the neurons had not been counted. This assumption was checked by repeating the analyses with the 25th and 75th percentiles and was found not to affect the results.

In order to determine which of the explanatory variables (diagnostic category, days affected and age) were important, and exactly how these variables were related to the severity of central pathology, data from the 58 EGS cases in which the majority of the four nuclei were available were analysed using generalised linear models and the software package GLIM 3.77 (Royal Statistical Society, London 1985). Generalised linear models were considered to be an appropriate tool for the analysis of the data in this study since, unlike standard linear regression models, they allow the inclusion of variables with normal and non-normal distributions. Models with binomial error structures were used to analyse proportion data without loss of statistical power and all predictor variables were fitted along with interaction terms. Overdispersion was corrected for using Williams correction (Crawley, 1993). For the proportion of nuclei, neurons and sections affected, variables investigated were the age of the animal, the diagnostic category of EGS and the duration of disease in chronic cases. The significance of model terms was assessed by stepwise removal from the corrected maximal model, comparing the change in residual variance to the Chi-squared distribution with the appropriate degrees of freedom. In order to determine which diagnostic categories had an effect on the model, the analysis was first repeated excluding data from AGS cases, and then repeated comparing data from SGS and CGS cases together with data from AGS cases. Data were analysed for all four nuclei together giving equal weights to all four nuclei.

f) Sensitivity of strategic central neuropathology as a post mortem diagnostic test

All data was re-evaluated to determine the sensitivity of a complete neuropathologic examination of the brain when used as a post-mortem diagnostic test for EGS.

g) Spinal cord neuropathology and the correlation between muscle trembling and LMN chromatolysis in EGS

A subjective grade on a scale of 0 (no trembling) to 4 (severe) was assigned to eight EGS cases, focusing on the appendicular musculature of the thoracic limb. Since the degree of trembling shown by a case at a particular time was found to be very variable, the greatest severity noted was used in the analysis.

At least one section of spinal cord, either C1 or C8, was removed from EGS 49 cases by isolating the vertebral column and exposing the spinal cord with parasagittal cuts using a band saw (Bursgreen, Wadkin ltd.). The spinal cord cervicothoracic intumescence was isolated and sectioned at it widest point, corresponding to the C8 segment. All multipolar cells with 3-5 concave sides and densely stained Nissl substance in the cytoplasm in both ventral horns were counted as lower motor neurons. In addition, all chromatolytic cells in the ventral horn were counted and noted as a proportion of all LMNs. The ventral horn was defined as the grey matter ventral to a straight horizontal line through the central canal (Polack *et al*, 1998).

The number of chromatolytic LMN neurons was reviewed in the eight cases in which a trembling score had been determined in order to determine if there was an association.

h) Lateral cuneate nuclei

The lateral cuneate nuclei had been noted to contain prominent spheroids in a pilot study, and have been reported to be involved in EGS (Gilmour, 1973b). Sections containing the lateral cuneate nucleus were re-examined in 13 EGS cases (7 AGS, 5 SGS and 1 CGS) and four control animals by two blinded observers and scored according to the degree of pathology. If the scores from the left and right lateral cuneate nuclei were different the higher score was used. Pathology was graded 0 (one or two isolated spheroids), 1 (more than two isolated, small spheroids), 2 (several large spheroids), 3 (substantial spheroid formation with vacuolation or fragmentation) or 4 (substantial spheroid formation with vacuolation and fragmentation, loss of cell bodies, prominent astrocytosis and lipofuscin deposits).

3. RESULTS

a) Case material

A significantly larger proportion (Chi Square: $p < 0.003$, Table 2-1) of cases included in this database had acute grass sickness compared to cases presented to the R(D)SVS in the previous 10 years. The proportion of chronic cases examined however was not significantly different ($P=0.14$). The true EGS case distribution is unknown, but it is likely that chronic cases at the R(D)SVS are over represented since these would be viewed as potentially treatable by referring practitioners.

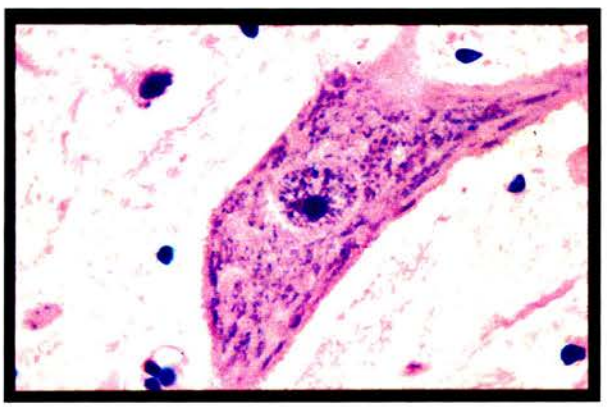
Table 2-1: Proportion of cases (N) in the three clinical categories of EGS included in this study

Current Study	AGS	0.48 (29)
	SGS:	0.22 (14)
	CGS	0.30 (18)
	Total	1.0 (61)
R(D)SVS database	AGS:	0.28 (105)
	SGS:	0.32 (117)
	CGS:	0.40 (147)
	Total	1.0 (369)

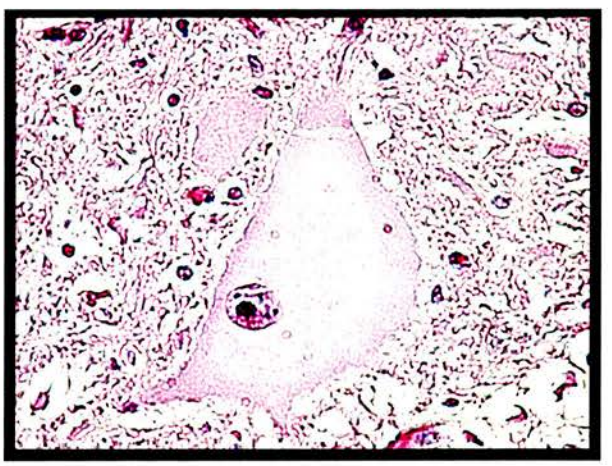
b) Descriptive pathology

(i) EGS Cases

Over 2000 observations were entered into the data base for analysis. Significant pathology was restricted to changes of the neuronal somata, which were swollen with pale pink cytoplasm lacking apparent Nissl substance and (often) containing a central or eccentric 'open faced' nucleus (Figure 2-4). An occasional chromatolytic neuron was seen in the reticular formation on some sections. More significantly, a proportion of LMN neurons in specific GSE and GVE nuclei in the brain (Figure 2-5) and spinal cord appeared markedly chromatolytic. Occasional neurons appeared to be undergoing central chromatolysis and rarely the nucleus was pyknotic. Evidence of neuronophagia or glial scarring was no more prominent in sections examined from EGS than in control cases. Small and medium sized axonal spheroids were frequently observed in the lateral cuneate nucleus. Three acute cases and one subacute case of confirmed EGS had no evidence of pathology noted in the brainstem, and in one further acute and one chronic case no central somatic neuronal pathology was noted. The material from these cases was re-examined but no further sections were cut. The occurrence of neuronal pathology is summarised for all cases of EGS in Table 2-1.



A.



B.

Figure 2-4: Normal (A) and chromatolytic (B) lower motor neurons, nucleus of CN XII, CGS

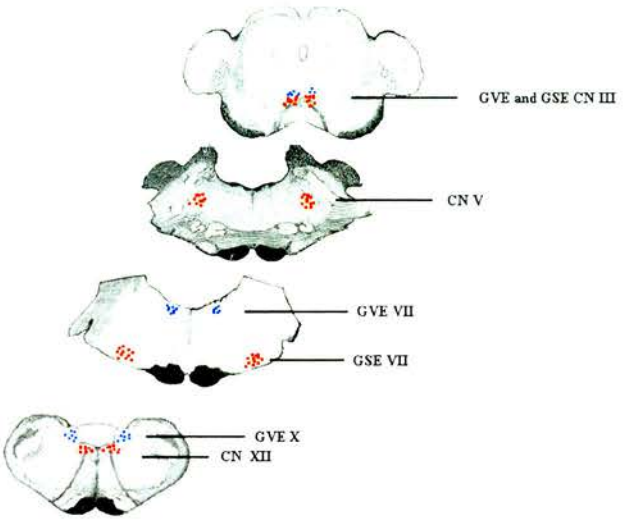


Figure 2-5: Diagram of the distribution of chromatolytic neurons in the nuclei of cranial nerves at representative levels of the brain. The proportion of neurons affected is not represented. Modified from de Lahunta (1983).

Table 2-2: Summary of neuronal pathology in EGS cases

Nucleus or Structure	Affected	Total	Prop Affected (95% CI)
Olfactory bulb	0	11	0 (0 - 0.28)
Temporal lobe cortex	0	34	0 (0 - 0.10)
Cerebellum	0	40	0 (0 - 0.09)
Basal nuclei – caudate, putamen, pallidum	0	34	0 (0 - 0.10)
Thalamus	0	31	0 (0 – 0.11)
GSE nucleus of CN III	36	62	0.58 (0.45-0.70)
GVE nuc. of CN III (Rostral GVE column)	7	16	0.44 (0.20 - 0.70)
Red nucleus	0	46	0 (0 - 0.08)
Substantia Nigra	0	38	0 (0 - 0.09)
Mesencephalic nucleus of CN V	2	60	0.03 (0 - 0.06)
Pontine Nuclei	0	42	0 (0 - 0.08)
Motor nucleus of V	21	54	0.39 (0.26 - 0.53)
Nucleus of CN VI	10	32	0.31 (0.16 - 0.50)
Cochlear nucleus	0	18	0 (0 - 0.19)
Vestibular nuclei	3	57	0.05 (0.01 - 0.15)
GSE nucleus of CN VII	28	55	0.51 (0.37 - 0.65)
Nucleus of the Trigeminal tract	0	52	0 (0 - 0.07)
Reticular formation	3	42	0.07 (0.01 - 0.19)
Olivary nucleus	0	33	0 (0 - 0.11)
Nucleus of GVE X (Caudal GVE column)	16	58	0.28 (0.17 - 0.41)
Nucleus of CN XII	20	58	0.34 (0.22 - 0.48)
Nucleus of the Solitary Tract	0	18	0 (0 - 0.19)
Lateral Cuneate nucleus	0	42	0 (0 - 0.08)
Spinal cord LMNs	17	49	0.35 (0.22 - 0.50)
Spinal cord ILH Neurons	15	18	0. 83 (0.59 – 0.96)

Total = number of cases in which the nucleus or structure was examined. The 95% confidence intervals (CI) were calculated from the Binomial distribution (Rohlf and Sokal, 1981).

c) Control animals

Very few lesions were recognised in control animals. Spheroids were occasionally noted in the reticular formation and particularly the lateral cuneate nucleus, and shrunken or dark staining neurons and neuropil vacuoles were infrequently described but were concluded to be processing artefact. A few small accumulations of mononuclear cells ('glial scars') were seen in the grey matter of the cortex of one case. With the exception of two chromatolytic cell bodies in the red nucleus of one control animal, no nuclear regions examined were found to contain chromatolytic neurons.

d) Consistency of pathology

(i) Agreement of the presence of pathology between repeated sections through a nucleus

A total of 185 sections containing more than one section each of the nucleus of CN III, V, VII, XII or GVE X were examined, of which 33 sections were classified as unmatched. There was no significant difference in the proportion of cases in which there was disagreement in the pathology of individual nuclei when either the data of different nuclei in all cases of grass sickness ($p > 0.25$), or the different severity categories of grass sickness for all the nuclei ($p > 0.5$) were compared by a Kruskal-Wallis one way analysis of variance on ranks. To evaluate the proportion agreement beyond chance, all the disagreement data were combined giving a moderate Kappa value for spatial repeatability of 0.59 (95% CI 0.45 to 0.74).

(ii) Consistency of the proportion of chromatolytic neurons noted on repeated sections through a nucleus

The proportion of chromatolytic neurons was determined on 28 repeated sections of four blocks of the nuclei of CN X and CN XII on one case, and from 19 sections of three blocks of the nucleus of CN VII from another (Figure 2-6). Mann-Whitney Rank Sum Tests comparing the first section in each block, the result of which were initially noted in the database, with the subsequent sections through that block, showed no significant difference in the 11 blocks examined ($p>0.1$). The data between blocks was compared using a one way analysis of variance on ranks and was not significantly different for the nucleus of GVE X ($p> 0.1$) or CN VII ($p>0.5$), but was significantly different for CN XII ($p> 0.02$). Tukey's multiple comparison procedures indicated that in that in the latter case there was a significant difference only between two of the three blocks .

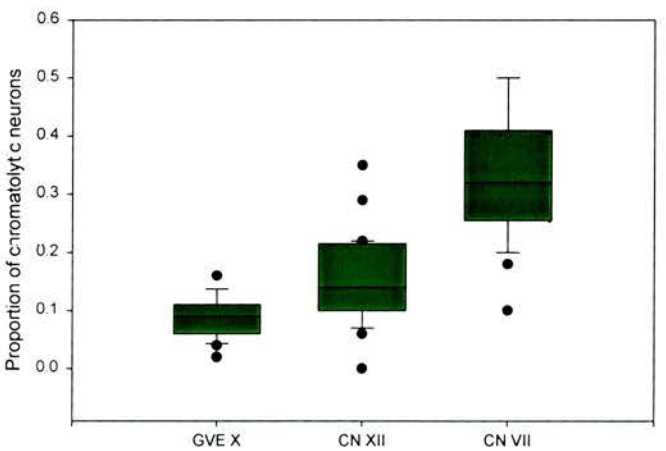


Figure 2-6: Repeated sections summary data
Key: The ends of the boxes define the 25th and 75th percentiles with a line at the median and error bars defining the 10th and 90th percentiles.

(iii) Consistency: GVE column

The number of neurons in the rostral GVE column was probably underestimated as it is likely that a number of the chromatolytic neurons noted as GSE in the oculomotor nucleus, are actually part of the Rostral GVE column. For the sake of consistency however only neurons in the very rostral part of the mesencephalon were included as part of the rostral GVE column.

There was poor agreement (Kappa = 0.23) between the presence of neuronal chromatolysis in the rostral and caudal portions of the GVE column, with disagreement on the presence of pathology (present in the caudal portion and absent in the rostral portion, or vice versa) in 11/13 cases.

e) Severity of brainstem somatic lower motor neuron pathology

Data from the somatic nuclei of CN III, V, VII and XII from 59 horses were thus analysed using general linear models. A total of 9319 neurons on 472 sections were examined. Data for all four nuclei were not available for each animal with 76%, 17% and 7% of cases having four, three and two nuclei noted respectively.

(i) Proportion of nuclei affected

Summary data of the proportion of cases with chromatolytic neurons noted in the nuclei of CN III, V, VII or XII for AGS, SGS and CGS cases are given in Figure 2-7. The proportion of the four nuclei containing chromatolytic neurons in cases included in the model differed significantly ($p<0.001$) between the diagnosis categories (Figure 2-8), and varied inversely and significantly ($p<0001$) with the age of the animal (Figure 2-9). The interaction between these terms was not significant ($p>0.05$) showing that the effect of age was similar in all three diagnosis categories. Further analysis revealed that the proportion of nuclei affected did not vary between SGS and CGS cases ($p>0.05$), and the proportion of nuclei affected in SGS and CGS cases was significantly ($p<0.001$)

higher than that in AGS cases. Duration of illness was analysed only for chronic cases, since acute and subacute cases were in the hospital for only short durations. This had a significant negative effect ($p<0.05$), but this was small compared to the effect of age.

(ii) Proportion of chromatolytic neurons

The proportion of all chromatolytic neurons in the nuclei of CN III, V, VII and XII differed significantly ($p<0.001$) between the diagnosis categories (Figure 2-8), and varied significantly ($p<0001$) and inversely with the age of the animal (Figure 2-9). The interaction between these terms was not significant ($p>0.05$). The proportion of nuclei affected did not vary between SGS and CGS cases ($p>0.1$), but was significantly higher than that in AGS cases ($p<0.005$). In a separate model containing only CGS cases, age had a marginally significant effect ($0.1>p>0.05$) and duration did not have a significant effect on the proportion of chromatolytic neurons present.

(iii) Proportion of sections with chromatolytic neurons

Both the diagnosis ($p<0.001$, Figure 2-8) and age ($p<0.001$, Figure 2-9), but not their interaction ($p>0.1$), showed a significant influence in the model. All the diagnosis categories were significantly different from each other (AGS, $p<0.001$; SGS and CGS, $p<0.05$). When only chronic cases were considered, age again had a significant negative effect ($p<0.005$) but duration was found to be only marginally significant ($0.05>p>0.025$).

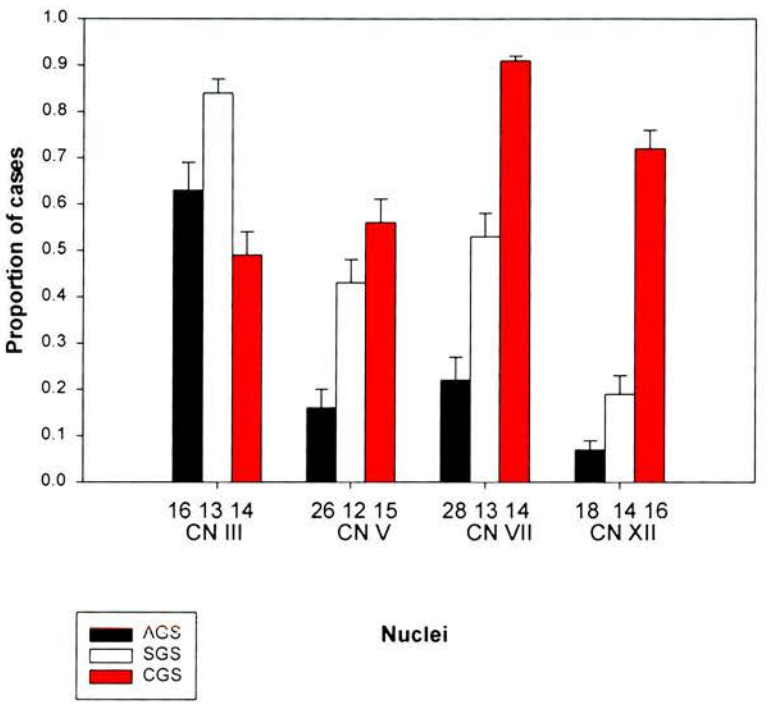
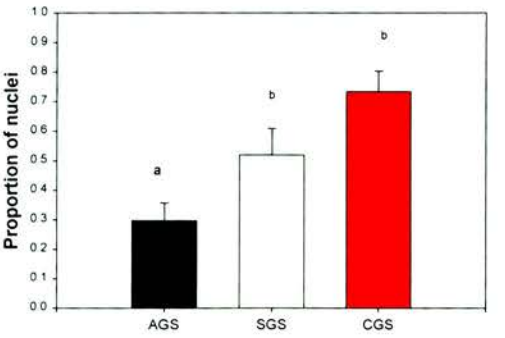
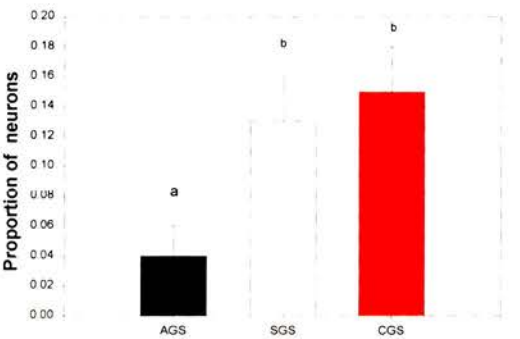


Figure 2-7: The proportion of cases with chromatolytic neurons noted in the nuclei of CN III, V, VII or XII for AGS, SGS and CGS cases.
Model mean plus 95% CI. The number of cases examined are given below the x axis.

(i) Proportion of nuclei



(ii) Proportion of neurons



(iii) Proportion of sections

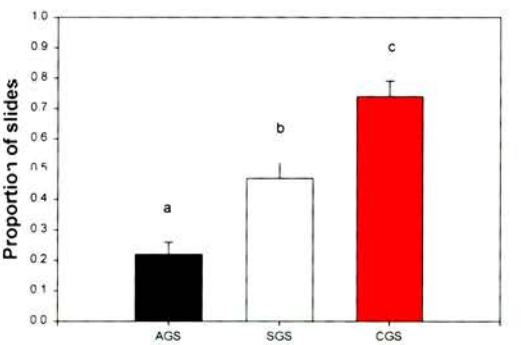
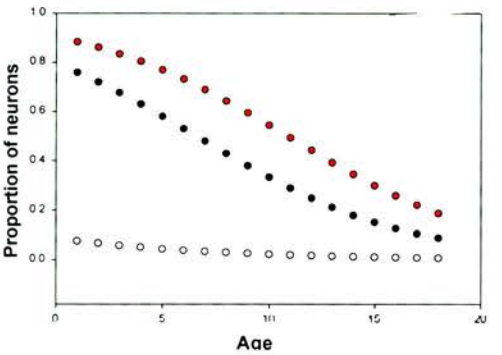
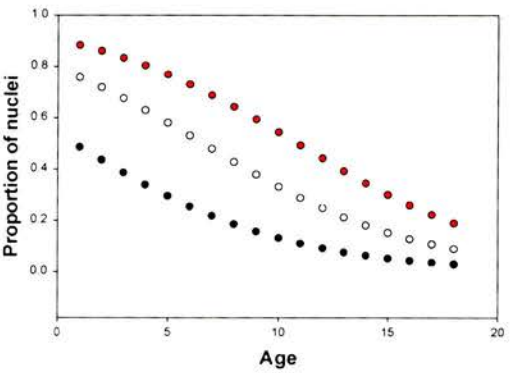


Figure 2-8: The proportion of neurons (i) nuclei (ii) and sections (iii) of CN III, V, VII and XII with chromatolytic neurons in AGS, SGS and CGS cases. Data plotted are model estimates + 1 S.E. The difference between different letters is statistically significant, $p < 0.001$.

(i) Proportion of nuclei



(ii) Proportion of neurons



(iii) Proportion of sections

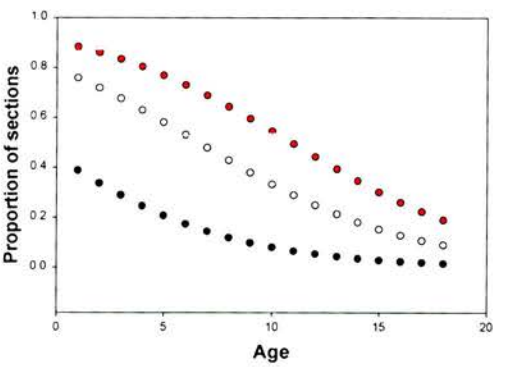


Figure 2-9: Influence of age on the proportion neurons (i) nuclei (ii) and sections (iii) of CN III, V, VII and XII with chromatolytic neurons in AGS, SGS and CGS casess. Points are model estimates.
Key: AGS - • , SGS - ○ , CGS - ●

f) Sensitivity of strategic central neuropathology as a post mortem diagnostic test

When all CNS sections from 58 EGS cases and 12 control animals were considered, 54 EGS cases had chromatolytic neurons noted on at least one CNS section in the GVE or GSE nuclear columns whereas four cases did not. No control animal had chromatolytic neurons noted in these nuclei. A sensitivity of 93% and specificity of 100% was thus derived for diagnosing EGS following a complete CNS histopathologic investigation.

g) Spinal cord pathology and the correlation between muscle trembling and LMN chromatolysis in EGS

Chromatolysis of preganglionic sympathetic neurons in the intermediolateral horn was noted in 83% of the 18 cases examined. GSE LMN chromatolysis was present in 35% of the 49 cases in which at least one section of spinal cord had been examined (Table 2-2).

The median trembling score was 2, range 1-3. Seven of the eight cases had no evidence of spinal cord LMN chromatolysis noted. One case showed evidence of chromatolysis in a small proportion of LMNs (0.06) and had a low trembling score (1). There appeared to be no correlation between trembling scores and spinal cord LMN chromatolysis.

h) Lateral cuneate nuclei

Case and severity score summaries are given in Table 2-3. If the scores from the left and right lateral cuneate nuclei were different the higher score was used. There was no statistical difference between the scores of EGS cases and control animals (Mann-Whitney Rank Sum Test, $p=0.1$).

Table 2-3: Pathology of lateral cuneate nuclei

	Number	Median age (range)	Median severity score (range)
EGS	13	3 (2-5)	0 (0-2)
Control	4	7.5 (2-8)	2 (0-1)

Key: 0 = one or two isolated spheroids, 1 = more than two isolated, small spheroids, 2 = several large spheroids

4. DISCUSSION

The distribution of central lesions in EGS appears to be very specific and repeatable, to the extent that strategic central neuropathology could be used as a sensitive and specific post mortem diagnostic test. This agrees with Barlow (1969) but contradicts the conclusions drawn by Wright (1988). The latter study, although it examined 11 cases, did not assess all nuclei in each case and may have been confounded by the nuclei not being diffusely affected throughout the GVE or GSE column. All previous studies that examined the brain of grass sickness (Barlow, 1969; Gilmour, 1973b; Wright and Hodson, 1988) and mal seco (Uzal *et al*, 1994) cases , reported pathology in one of the vestibular nuclei. Only three cases in the current study had a few chromatolytic neurons noted in the vestibular nuclei on careful examination of 58 cases, the neuroanatomic identity of affected nuclei having been confirmed by specialists in the field. It may be that the authors of the previous papers had mistaken neurons of the motor nucleus of CN V for a vestibular nucleus, since the former have similar sized neurons and are placed only slightly further rostral to the latter.

The distribution of central pathology in EGS in this study is unlike that reported in any other equine or human disease, however it appears to be the strikingly similar to that in cats (Griffiths *et al*, 1982), dogs (Schultze *et al*, 1997) and hares (unpublished observations) with primary dysautonomias. Unlike the horses in this study however, chromatolytic neurons were not seen in cases of cats with dysautonomia with disease duration of longer than 2 weeks.

Similarly to EGS, the nuclei of the hypoglossal, facial and trigeminal motor nuclei are also regularly affected in amyotrophic lateral sclerosis in humans (Blackwood *et al*, 1964; Esiri, 1996) but the oculomotor, trochlear and abducens nuclei are not. It has been observed however that in cases in which respiratory support has prolonged life of human patients by delaying death from respiratory failure, nuclei regarded as resistant to disease, including the oculomotor nuclei, have been found to be affected. This may imply that the selective vulnerability of a functional system is a relative phenomenon and, given time, the changes become more widespread (Ince *et al*, 1998). A similar process may account for the finding that the somatic nucleus of CN X was not noted to contain chromatolytic neurons, despite representing a large portion of the caudal somatic efferent column and being affected in cats (Pollin and Griffiths, 1987) and hares (unpublished observations) with dysautonomia. The 'nucleus ambiguus' is notoriously difficult to distinguish from other nuclei in the reticular formation, however it would have been registered if chromatolytic neurons had been present.

A specific anatomic distribution of neuropathology is a feature of a number of human and animal neurodegenerative diseases, suggesting that the neurons in affected nuclei share a common factor making them sensitive to specific insults. Individual neurons differ greatly from each other in architecture, neurotransmitter production, state of functional activity and metabolic requirements. A specific shared feature, such as metabolic activity, unique to a pool of neurons, may make these neurons selectively vulnerable to a particular insult (Waggie *et al*, 1999) and solving this intriguing question may greatly improve the opportunity to determine the respective aetiologies.

Lesions in the motor nuclei of the trigeminal, facial and hypoglossal nerves are also a consistent features of EMND (Cummings *et al*, 1990), however the lack of pathology in the nucleus of CN III in that disease and the absence of the punctate eosinophilic neuronal inclusions (Cummings *et al*, 1993) or evidence of neuronal death in the CNS

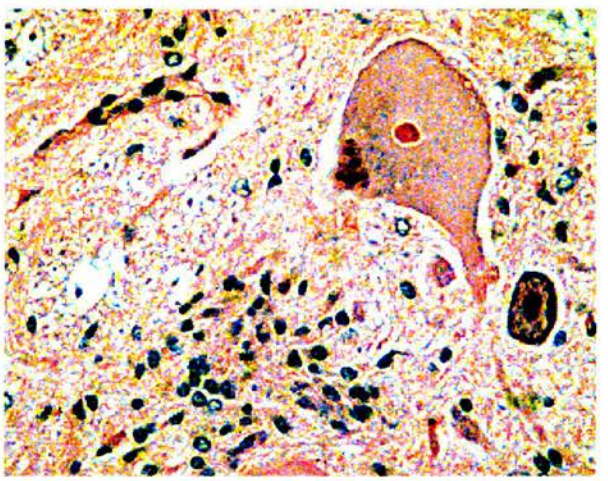


Figure 2-10: Chromatolytic motor neuron, equine motor neuron disease. Note cytoplasmic inclusions in the soma of the nucleus of cranial nerve VII next to an accumulation of glial cells removing the remainder of a necrotic neuron (H&E x 400).

A further feature dividing EGS from classical human neurodegenerative diseases (Schwartz *et al*, 1999), is the absence of an invariable increase in severity of pathology and clinical signs with increased duration of disease. The changes in central neuronal pathology in EGS cases have been described as being degenerative in character and to become more severe with increasing duration (Barlow, 1969). However the current study does not support this. The small statistical trend for decreasing severity of brain pathology with increased duration of disease may suggest somatic neurons are recovering from the initiating insult. In the absence of a clinical model however it is not possible to determine whether those animals had less severe disease from the outset. Sections from the brain of one animal examined many years after recovering from EGS (unpublished observations), had a marked depletion of peripheral postganglionic sympathetic neurons but did not have evidence of loss of central neurons or formation of glial scars, as was also the case in this study, suggesting that central neuronal

(unpublished observations), had a marked depletion of peripheral postganglionic sympathetic neurons but did not have evidence of loss of central neurons or formation of glial scars, as was also the case in this study, suggesting that central neuronal recovery occurs in time. The presence of degenerating neurons and neuronophagic nodules however has been reported in cats with dysautonomia (Pollin and Griffiths, 1987), which implies a difference in the cellular pathology despite the similarity in the distribution of affected neurons.

More difficult to explain is the clear association in this study between increased severity of central neuronal pathology and less severe clinical disease; AGS cases having fewer chromatolytic central neurons than CGS cases. This opposes observations by Gilmour [11] who recorded a greater severity in acute cases. The durations of disease were not equivalent between diagnostic categories and it would be interesting to establish whether acute cases with longer durations have a severity of central neuropathology equivalent to chronic cases at the same duration of disease. This study would be extremely difficult to perform because determining the severity of enteric neuronal pathology necessitates early euthanasia in the former group of cases.

The inverse association of age and severity of brain pathology by all criteria examined had not been recognised prior to obtaining the results of the statistical models. It is known that younger adult animals are predisposed to EGS (Gilmour and Jolly, 1974; Doxey *et al*, 1991a; Wood *et al*, 1998) with a later comprehensive epidemiological study (Wood *et al*, 1994) finding that 56% of EGS cases were five years or younger. However, there is no difference in ages between animals affected with acute, subacute or chronic disease which have been presented to the University of Edinburgh in the last ten years, suggesting that age is not associated with the clinical severity of peripheral neuronal pathology.

The lack of correlation between trembling and spinal cord lower motor neuron soma pathology in EGS implies that this clinical sign is unlikely to be due to LMN paresis but

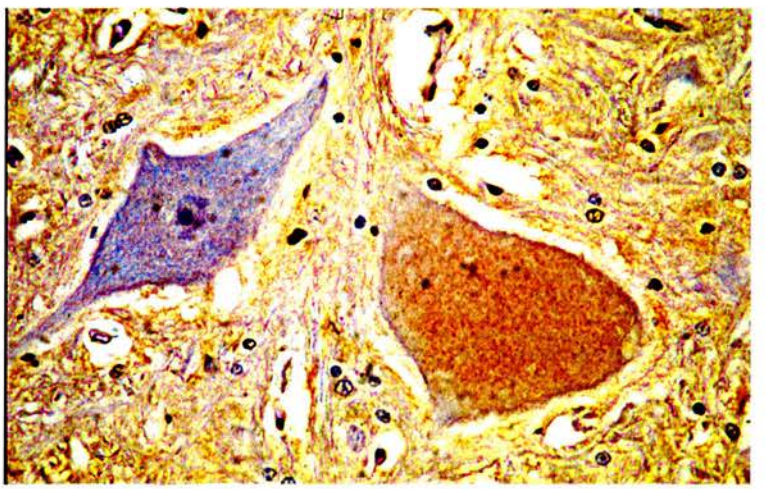
may be a physiological tremor. The characteristics of the trembling differs from those invariably seen in equine motor neuron disease (Divers *et al*, 1992; Kuwamura *et al*, 1994), the tremors in EGS consisting of finer, higher frequency fasciculations extending over the axial musculature and continuing when the horse is recumbent. Fasciculations can be caused by a variety of lesions to peripheral structures from the nerve root to the neuromuscular junction (Lance, 1988; Desai and Swash, 1997, Layzer, 1994). Higher frequency physiological tremor such as 'essential tremor' is common in humans and can be modified by cortical input (Teravainen and Larsen, 1984; Comby *et al*, 1992) but the anatomical origin of the abnormal discharges is not known (Simpson and Thomaides, 1988). Electrodiagnostic techniques may be required to determine if the trembling is due to the activity of individual muscle fibres (fibrillations), whole motor units (fasciculations) or consists of undulation of entire muscle bellies (myokymia) (Lance, 1988) and to attempt to determine where they originate (Behnia and Kelly, 1991; Layzer, 1994).

A structure frequently reported to be abnormal in EGS cases is the lateral cuneate nucleus. The abnormalities noted were almost certainly spheroids, which represent focal distensions of axons with organelles that normally are in transit away from or towards the soma and are a non-specific response to many diverse insults. They are encountered with increasing frequency with progressive age (Summers *et al*, 1995) and are a diagnostic characteristic of equine degenerative myeloencephalopathy (Mayhew *et al*, 1977), a disease that is associated with vitamin E deficiency and may have a familial component (Beech, 1994). An increased frequency of spheroids in the lateral cuneate nucleus has also been noted in cases of equine idiopathic laryngeal hemiplegia, a disease characterised by distal axonopathy of the recurrent laryngeal nerve (Cahill and Goulden, 1989). In the absence of a case control study however, as was undertaken here, it can be difficult to determine if the presence of spheroids is within normal ranges for the age of the animal, and this may account for the observation of pathology in this structure by previous authors.

In conclusion, the consistent finding of somatic neuronal chromatolysis in EGS suggests that, despite the lack of apparent somatic neuronal cell death or overt clinical evidence of clinical somatic involvement, this disease should be classified as a multiple system disorder. The involvement of multiple systems is increasingly found to be a feature of distinct human neurodegenerative disorders, such as Parkinson's disease (Braak *et al*, 1995), and it has been speculated that the involvement of multiple systems may be inevitable if the disease process is of a sufficiently long duration (Takahashi *et al*, 1993; Sato *et al*, 1995). In familial dysautonomia however, a human disease which appears to share many characteristics of EGS, there in no evidence of specific brainstem somal neuronal pathology (Fogelson *et al*, 1967; Pearson *et al*, 1971) despite clinical (Tonholo-Silva *et al*, 1994) and electrodiagnostic (Lahat *et al*, 1992) evidence of motor and brainstem dysfunction. The similarity of the distribution of central neuropathology to the primary dysautonomias in cats, dogs and hares is further incentive to search for a common aetiologic agent

Chapter Three

Central Cytopathology



1. INTRODUCTION TO CYTOPATHOLOGY IN EGS

The morphology of neurons in EGS was comprehensively described in Obel's seminal description of 'Grass Disease' in 1955 . Ultrastructural studies followed (Chandler and Brownlee, 1967; Gilmour, 1975; Hodson *et al*, 1984b) but little molecular work detailing the cytopathology has been accomplished and has focused solely on enteric and peripheral sympathetic neurons.

Sabate *et al* (1983) investigated gut regulatory peptides in EGS and found a great reduction in the numbers of nerves containing VIP, substance P, bombesin and enkephalin, particularly in the ileum, and fewer than normal somatostatin and enteroglucagon containing cells. Very similar changes were noted by Bishop *et al* (1984). Murray *et al* (1994), in a functional study, recorded muscarinic receptor hypersensitivity to bethanechol by strips of smooth muscle from AGS cases. Peripheral sympathetic ganglia were studied by Griffiths *et al* (1993), noting a marked disturbances in neurofilament proteins and beta-tubulin with severely reduced staining for neuron-specific enolase, the catecholaminergic enzyme tyrosine hydroxylase and an increase in dopamine-beta-hydroxylase, another enzyme associated with noradrenaline synthesis. Membranes of the Golgi apparatus were histochemically stained and revealed a profound loss of a recognisable Golgi structure in affected neurons. Postganglionic sympathetic ganglia in EGS and mal seco cases were additionally shown to contain neurons staining for whole horse serum, which may occur non-specifically following damage to the cell membrane (Griffiths *et al*, 1994b; Giruaudo Conesa and Uzal, 1996).

The principal abnormality noted in central neurons in EGS is chromatolysis, with an extensive loss of defined Nissl substance (Nissl, 1894), often an eccentric nucleus with a large nucleolus and a marked enlargement of the perikaryon. The latter is unlike neurons in ALS, in which chromatolytic motor neurons have been found to be about 50% smaller than those in non-ALS patients with chromatolysis due to nerve root pathology

(Wakayama, 1992). Classically, chromatolysis is seen a few days after axonal transection and appears to be a regenerative response coinciding with an increase of protein synthesis and axonal regeneration (Weller, 1984). The hallmark appearance of the cytoplasm is due to dispersal of Nissl bodies, stacks of rough endoplasmic reticulum and intervening groups of polyribosomes present in perikarya and dendrites (Peters *et al*, 1976). The nature of the trigger is controversial (Thomas *et al*, 1993).

The term 'chromatolysis' (chrom- [Gr] *colour*) is by some authors defined strictly as a decrease or loss of cytoplasmic basophilia in the perinuclear region of the neuron associated with axonal injury, while neurons that are pale and swollen for other reasons have been referred to as 'ballooned' neurons or neurons showing achromasia (Greenfield *et al*, 1988). Examples of diseases marked by the presence of ballooned neurons are relatively rare in human and veterinary neuropathology, and veterinary pathologists have tended to refer to the latter cell as 'chromatolytic' while the former change is termed the 'axonal reaction' (Summers *et al*, 1995). The word chromatolysis in this thesis is used merely to describe the morphology of the cell and not the aetiology of the loss of Nissl staining.

Cytopathology is studied to determine the aetiopathogenesis of the disease, and thus limit the number of potential insults. Individual neurons differ greatly from each other in architecture, neurotransmitter release, state of functional activity and metabolic requirements (Waggie *et al*, 1999), and information about specific shared features unique to a pool of neurons making these neurons selectively vulnerable to a disease may further narrow the field. The initial investigation of the cytopathology of central neurons in EGS is the basis of this chapter.

2. Neuronal Cell death

a) Introduction and literature review

An issue which appears fundamental to the study of central neuronal cytopathology in EGS cases, is whether affected cells are dying or are likely to recover from the insult. This very basic question requires an understanding of the two types of cell death that are now recognised, apoptosis and necrosis.

Apoptosis, variously referred to as 'cell suicide' or 'programmed cell death', was discussed in the literature as early as 1972 (Kerr *et al*,), replacing the previous concept of 'necrobiosis' or 'single cell necrosis', however it did not receive a great deal of scientific attention until the late 80s. To date there are over 35,000 publications on this topic with around 600 papers referring to apoptosis published per month.

The basic process of apoptosis has been well reviewed (Raff, 1992; Schwartzman and Cidlowski, 1993; Columbano, 1995; Levin, 1995; Vaux and Hacker, 1995; White, 1996). Classically, apoptosis occurs physiologically by a process encoded in the genome in order to regulate numbers of cells during development, after hyperplasia, after cell senescence, as a defence mechanism to limit the replication of intracellular parasites and in response to oxidative stress (see Appendix 3).

Reactive oxygen species are hypothesised to be intimately involved in the process of apoptosis, both in the induction phase and in the final degradation phase. Much emphasis has been placed on the excitotoxic amino acid glutamate and its interaction with N-methyl-D-aspartate (NMDA) type channels (Nicotera *et al*, 1997). It is speculated that reactive oxygen species generated by glutamate, and opening of NMDA activated calcium channels, induce an increase in mitochondrial permeability leading to apoptosis (Kroemer *et al*, 1997) or, if exposed to high concentrations, a loss of mitochondrial membrane potential and necrosis (Ankarcrona *et al*, 1995). A substantial

amount of evidence now exits that excitotoxicity leads to acute necrosis and delayed apoptosis (Pang and Geddes, 1997).

Apoptosis can also be induced pathologically following damage by chemicals, microorganisms or ischaemia and is the mode of target cell death mediated by cytotoxic T lymphocytes and natural killer cells. Apoptosis is an active process of cell destruction of single cells characterised by a rapid course of early internucleosomal double-stranded DNA fragmentation into 180 bp DNA fragments, chromatin aggregation, nuclear pyknosis, cell shrinkage, and membrane blebbing. The cell eventually fragments into membrane bound acidophilic globules (apoptotic bodies) often containing organelles and nuclear material. These apparently provide a potent stimulus for phagocytosis by phagocytic cells or even neighbouring epithelial, endothelial or tumour cells. Inflammation is not elicited .

Details of the intracellular processes involved in apoptosis are probably still not complete and the identity of obligate mediators remains controversial. Consistent though is the cleavage of double stranded DNA into principally fragments with single base 3' OH overhangs (Mundle *et al*, 1995). This feature is the basis of the standard in apoptosis *in situ* detection techniques (Gavrieli *et al*, 1992; Ansari *et al*, 1993), terminal deoxynucleotidyl transferase (TdT) mediated UTP nick end labelling (TUNEL). This is reported to be a rapid, sensitive, and reproducible in situ assay of DNA fragmentation capable of detecting apoptosis in even very old formalin-fixed and paraffin embedded material (Bardales *et al*, 1997). The procedure labels DNA strand breaks in apoptotic cells with a non-isotopic reporter molecule (dUTP) in the presence of a DNA polymerase such as TdT, with immunohistochemical identification of labelled DNA. TdT is a template independent DNA polymerase adding deoxynucleotides to a 3'-OH terminus on single stranded or double stranded DNA. The 3'-OH acceptor can be an overhang, blunt or recessed end, although an overhang end is most efficient (Brown, 1991). Since apoptosis was originally defined by morphological criteria however, using

either electron or light microscopy, these remain the gold standard for documentation of apoptosis (Ankarcrona *et al*, 1995; Levin, 1995).

Necrosis (reviewed by (Buja *et al*, 1993; Schwartzman and Cidlowski, 1993; Columbano, 1995; Nicotera *et al*, 1997; Perry *et al*, 1997) on the other hand generally occurs in a group of adjacent cells and is characterised by the disappearance of membrane pumping activities resulting in mitochondrial, endoplasmic reticulum and cell swelling. Intense mitochondrial damage follows with rapid energy loss and electrolyte changes including increases in magnesium, chloride, sodium and most significantly calcium ions as well as potassium ion efflux. Lysosomes rupture causing cellular disintegration and DNA is either not fragmented or nonspecifically degraded into a continuous spectrum of sizes resulting in a diffuse smearing in gels. Membrane lysis follows, releasing intracellular constituents that evoke an inflammatory reaction. Its is typically a process associated with energy depletion but in some situations may be prevented by inhibitors of protein synthesis (Popp *et al*, 1978). There are no specific histochemical tests that identify necrosis but the early increased membrane permeability can be exploited in living cells by dye exclusion methods.

'There is no field of basic cell biology and cell pathology that is more confusing and more unintelligible than is the area of apoptosis versus necrosis' (Farber, 1994). The search for the presence of apoptosis in diseased tissues has highlighted that, despite fundamental appearing differences between the two modes of cell death, they can be difficult to differentiate. Many tissues and pathogenic processes contain necrotic areas as well as cells undergoing apoptosis (Columbano, 1995) in which case gel electrophoresis may not be sensitive or specific enough to confirm the presence of apoptosis. This is complicated by the fact that even apoptotic tissues will undergo secondary necrosis if the phagocytic system is overwhelmed (Manjo and Joris, 1995; Perry *et al*, 1997) and the finding that morphologically necrotic cells are also labelled using the TUNEL procedure (Gold *et al*, 1994; Charriaut-Marlangue and Ben-Ari, 1995; Levin, 1995; Mundle *et al*,

1995; Frankfurt *et al*, 1996; van Lookeren Campagne and Gill, 1996; Pang and Geddes, 1997). Necrotic or apoptotic cells may be differentiated by the difference in staining intensity (Mundle *et al*, 1995), perhaps because apoptotic cells contain a higher proportion of 3' overhangs, preferentially labelled by TdT.

Recent evidence of the existence of a morphological continuum of necrosis and apoptosis confuses the issue even further since it contradicts the premise that the two forms of cell death are distinct and mutually exclusive. Serum withdrawal from AKR-2B mouse fibroblast cells activated a succession of events followed by time-lapse video microscopy and electron microscopy (Simm *et al*, 1997) which were initially similar to those defined as apoptosis, i.e. nuclear condensation and membrane blebbing. This however was rapidly followed by cell lysis and disruption of mitochondria, both of which are characteristic of necrosis. Plasma membrane blebbing and mitochondrial damage in the absence of DNA fragmentation was also recorded after glutamate induced cell death (Tan *et al*, 1998) while simultaneously apoptotic DNA laddering and ultrastructural morphologic evidence of necrosis was found to occur in excitotoxic neuronal death in neurons in the adult (Portera-Cailliau *et al*, 1997a) and immature (Portera-Cailliau *et al*, 1997b) brain. It has also been suggested that DNA fragmentation in certain brain regions in Alzheimer disease may reflect metabolic disturbances in the premortem period, but that cell destruction is mediated through necrosis rather than apoptosis (Stadelmann *et al*, 1998).

Nevertheless, it is thought that apoptosis may be involved in many acute and chronic neurodegenerative diseases (Linnik, 1996) which would have implications for both the pathophysiology and the development of therapeutic agents. Unfortunately it is difficult to determine to what extent chronic neurodegenerative diseases involve neuronal apoptosis due to the low rate of cell death and therefore the small percentage of cells potentially undergoing apoptosis, the rapidity of apoptotic cell death with phagocytosis and the nonspecificity of TUNEL techniques in differentiating end stage apoptosis from

necrosis (Bredesen, 1995). Diseases such as Parkinson's disease have studies confirming (Mochizuki *et al*, 1997) and refuting (Kosel *et al*, 1997) the presence of apoptotic neurons. Similarly apoptosis may play a role in Alzheimer's disease but the value of blocking it is not yet known (Barinaga, 1998). In amyotrophic lateral sclerosis (ALS) however there is convincing *in vitro* (Alexianu *et al*, 1994), *in vivo* (Yoshiyama *et al*, 1994; Troost *et al*, 1995) and mouse model (Friedlander *et al*, 1997) evidence that apoptosis is involved in the death of ventral horn lower motor neurons and glial cells.

One of the purposes of this study was to determine whether apoptosis plays a significant role in the cell death of neurons in EGS, in the hope that the mechanism of cell death may help determine the nature of the aetiologic agent. Unlike in the central tissues, extensive histological evidence of cell death is found in postganglionic sympathetic ganglia, and this tissue was used for the initial investigation. In the second phase the technique was applied to central tissues. The apparent low specificity for apoptosis of the TUNEL technique was acknowledged, in the final analysis however regardless of what mode of cell death results in the DNA laddering, the TUNEL technique can be used as a method to identify dying cells (Portera-Cailliau *et al*, 1997b) and this was the principal goal when applied to central tissues.

b) Materials and Methods

(i) Subjects

The initial investigation focused on sections of cranial cervical ganglia. Sections from two AGS, six SGS, three CGS and seven control cases had TUNEL reagents applied to them. More than one section was examined from the majority of cases at different times. One positive control section of virally infected mouse brains, one section of sheep thymus and at least one DNAse positive control slide was included in every run.

In the next phase, 16 sections of central tissues in which chromatolytic neurons were present on H&E evaluation were included from ten EGS cases (Table 3-1). One section of equine thymus was included as a positive control section for each run

(ii) Laboratory methods

In the first phase, DNA laddering in the cranial cervical ganglia sections was detected using a standard TUNEL procedure. Five micron sections of formalin fixed paraffin embedded tissue on Vectabond slides were deparaffinised in xylene, rinsed in 100% ethanol and placed in 0.3% H_2O_2 in methanol at room temperature for 30 minutes. The sections were hydrated to water through graded alcohols and permeabilised with 1% Triton-X100 for 1.5 minutes. After rinsing in water the sections were digested in 10 µg/ml proteinase K in 20 mM Tris and 2 mM $CaCl_2$, pH 7 for 20 minutes at 37°C and placed in 0.01 M phosphate buffered saline (PBS). Positive control samples were equilibrated with DNase 1 buffer (30 mM Tris pH 7.2; 140 mM sodium cacodylate; 4 mM $MgCl_2$, 0.1 mM dithiothreitol) for 20 minutes at room temperature and incubated for 30 minutes at 37°C in this buffer and 5 µg/ml DNase 1. All specimen were equilibrated with TUNEL buffer (30 mM Tris pH 7.2; 140 mM sodium cacodylate; 1 mM cobalt chloride) for 20 minutes at room temperature and incubated with 0.6 units/µl TdT enzyme (terminal deoxynucleotide transferase, Promega) and 20 µM Digoxigenin-

11-dUTP (Boerhinger Mannheim) in TUNEL buffer for 2 hours at 37°C in a humidified container. Sections were blocked with 3% normal goat serum and incubated with 1:100 sheep anti-digoxigenin peroxidase labelled Fab (fragment antigen binding) fragments (Börhinger Mannheim). Negative control samples had either the TdT enzyme or the digoxigenin labelled dUTP omitted. The sections were rinsed with PBS and incorporated DIG-dUTP detected by reacting with 3' diaminobenzidine (Peroxidase Substrate Table Set, Sigma Immuno Chemicals) for 20 minutes at room temperature. The reaction was stopped by rinsing in distilled water. The sections were counterstained with haematoxylin for 45 seconds, dehydrated and mounted.

In the second phase, the TUNEL procedure was applied to central tissues using a commercial kit (ApopTag – plus, Oncor). Five μm sections were placed on electrostatically charged slides (ProbeOn Plus, FisherBiotech) and deparaffinised in xylene, rehydrated through graded alcohols and placed in 10 μg/ml proteinase K/Tris/$CaCl_2$ for 30 minutes at 37°C to facilitate penetration. After rinsing in water, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water for 5 minutes. A positive control section was produced by incubating one tissue section in DNase 1 buffer at room temperature for 20 minutes before incubating with 5 μg/ml DNase 1 in buffer for 30 minutes at 37 ° C. After washing in PBS, all slides were immersed briefly in equilibration buffer and subsequently were incubated with working strength TdT Enzyme for 1 hour at 37° in a humid chamber. One negative control section had the TdT enzyme omitted. The reaction was stopped using the Stop/Wash Buffer, the sections were washed in PBS and had anti-digoxigenin peroxidase conjugate applied for 30 minutes at room temperature. Sections were placed in PBS peroxidase substrate for 3 - 6 minutes, washed in distilled water, counterstained with haematoxylin, dehydrated and mounted.

c) Results

The TUNEL assay proved to be problematic in its reproducibility and much time was spent trying to standardise and optimise the system. The results below refer only to those experimental runs from on which negative controls were negative and positive control sections, including mouse brain, thymus and DNAse treated sections, were labelled (Figure 3-1)

In phase one, more than one cranial cervical ganglion section was included from the majority of cases on each of the different runs. A few cranial cervical ganglion sections from EGS and control sections had labelled nuclei on some experimental runs. These usually appeared pyknotic (as opposed to open faced) and were in neurons with less cytoplasm than other cells (Figure 3-2). Sections which included labelled neurons on some sections but not on others had TUNEL reagent applied again in the second phase, but with the exception of a few positive glial cells, none were subsequently positive. In phase two no neurons from CNS sections were labelled. Microscopic examination of sections confirmed that chromatolytic neurons with nuclei were present.

Table 3-1: Central tissue TUNEL procedure

Case #	Diagnosis	Structure on section
97/1032	SGS	Nucleus CN V, XII & GVE X
95/387	SGS	C1 LMNs
96/396	SGS	Nucleus CN III & V
96/291	SGS	Nucleus III & VII
98/62	CGS	C8 LMNs
97/376	CGS	Nucleus CN VII, XII & GVE X
98/244	CGS	L6 LMNs
98/264	CGS	C8 LMNs
97/538	CGS	Motor nucleus V
97/293	CGS	Nucleus CN III

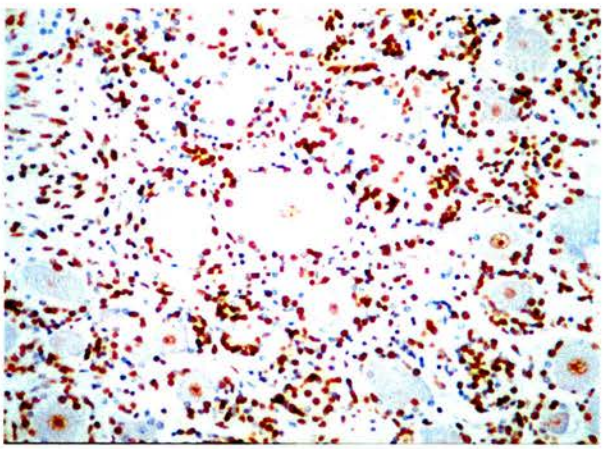


Figure 3-1: DNAse treated equine dorsal root ganglion positive control section. TUNEL procedure.

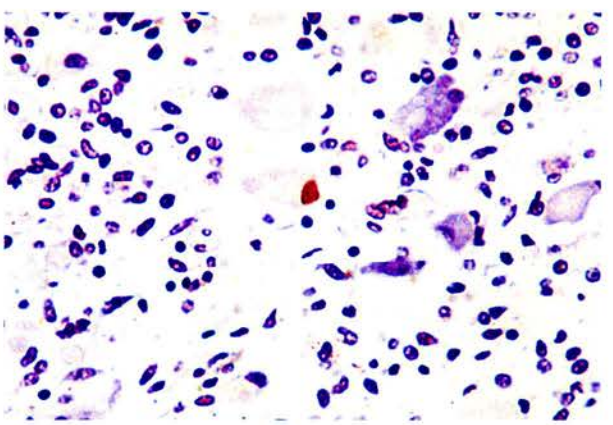


Figure 3-2: TUNEL positive postganglionic sympathetic neuron, cranial cervical ganglion, AGS.

d) Discussion

The observation that large numbers of neurons in the cranial cervical ganglia of EGS cases appeared to be dying in the absence of a marked inflammatory response (Brownlee, 1965), initially suggested that apoptosis may be the principal form of cell death. This was further supported by the suspicion that oxidative stress may contribute to the aetiology of EGS, due to the disease being associated with climatically stressed vegetation (B. McGorum, R(D)SVS, personal communication). The absence of overt apoptotic bodies being noted on routine histological sections is not unusual even in the presence of apoptosis, since TUNEL -positive cells are known to be cleared from the CNS by phagocytosis within 2 hours (Wetts and Vaughn, 1998). Some practice is needed to identify apoptotic bodies with the light microscope, and finding even relatively few of them on histological sections of a tissue implies that quite extensive 'drop out' is taking place (Kerr *et al*, 1972).

Nevertheless, and despite the occasional TUNEL positive cell being evident, it is reasonable to conclude that apoptosis is not a prominent form of cell death in EGS postganglionic neurons. Further work has been unable to show that oxidative stress is a significant component of the disease process in EGS (B. McGorum, R(D)SVS, personal communication), and autonomic neurons have been shown to be relatively resistant to excitotoxic cell death, which is thought to account for the lack of GVE neuronal pathology in the presence of somatic LMN death in amyotrophic lateral sclerosis (Wetts and Vaughn, 1998).

The TUNEL technique was applied to central tissues in order to label neurons that were dying, regardless of the underlying process. The sensitivity of the technique is arguably low, given that comparatively few chromatolytic central neurons with nuclei present on a tissue section. This decreased the probability of the appropriate fragmented DNA being present, particularly in view of the rapid removal of apoptotic cells. In addition, the specificity of the presence of DNA laddering for cell death is now questioned; recent

work has shown that there is evidence to suggest that TdT labelled cells may recover and not undergo terminal apoptosis. 70 - 80% of nuclei in entorhinal cortex/hippocampal formation in Alzheimer's disease show increased TdT labelling even early in the disease, far exceeding the actual loss of cells observed cases with longer disease duration (Cotman, 1998). The large number of cells with DNA fragmentation in some cases may reflect metabolic disturbances in the pre-mortem period and not indicate impending cell death (Stadelmann *et al*, 1998).

The lack of TUNEL staining in addition to the absence of histological evidence of somatic neuronophagia or glial cell accumulations, strongly suggest that the chromatolytic central neurons in EGS are capable of recovering. One could speculate that a metabolic component of somatic LMNs is able to defend the cell from a terminal insult of the putative EGS 'toxin', or that the CNS represents a 'protected' area able to shield those neurons. The similarity in the distribution of neuronal pathology between EGS and feline dysautonomia suggests that a very similar insult is involved in the disease in those two species, however the death of central neurons described in the latter (Pollin and Griffiths, 1987) may point away from that. The difference may represent a species specific effect, alternatively the agent may be modified by the nature of the putative source (Grass vs cat food??).

3. UBIQUITIN EXPRESSION IN GRASS SICKNESS

a) Introduction and literature review

Numerous, round, argyrophilic, 5 - 15μm eosinophilic bodies are present in perineuronal supporting tissue and in recesses in perikarya of normal and degenerate postganglionic sympathetic neurons of EGS cases. These closely resemble Parkinsonian Lewy bodies (Figure 3-3) and are present in the majority of chronic and subacute grass sickness sympathetic ganglia and very occasionally in central neurons. Gilmour (1975) noted on ultrastructural examination of EGS postganglionic sympathetic neurons that these contained mitochondria and vacuoles in vesiculotubular material, and were surrounded by a single limiting membrane and a sheath of satellite cell processes. It was proposed that they represent dystrophic axons, as similar changes have been described in experimental and infantile neuraxonal dystrophy in a number of structures including in the soma of nuclei of CN III and VII and spinal cord LMNs (Cowen and Olmstead, 1963). That work found no histopathological indications of axonal spheroids in any other central or peripheral structures in EGS, and it was proposed that these bodies may more likely be similar to Parkinsonian Lewy bodies.

Lewy bodies are an obligate characteristic of Parkinson's disease, although they are also occasionally found in multiple system atrophy, progressive supranuclear palsy, amyotrophic lateral sclerosis, Alzheimer's disease, Down's syndrome, Lewy body dementia and others. Two types are described: Classical (brainstem) and cortical (Lowe *et al*, 1997).

Classical Lewy bodies are intraneuronal, rounded, 8-30 μm eosinophilic inclusions with a hyaline core and a pale staining peripheral halo. In Parkinson's disease they are found in the substantia nigra, locus ceruleus and thalamus, hypothalamus, substantia innominata (basal forebrain nuclei) and parasympathetic nuclei of the vagus nerve. One neuron may contain many Lewy bodies. The halo has not been fully characterised but

associated with a granular electron-dense coating material and vesicular structures. It contains ubiquitin and proteins involved in ubiquitin metabolism. The core contains densely packed filaments associated with compacted granular material.

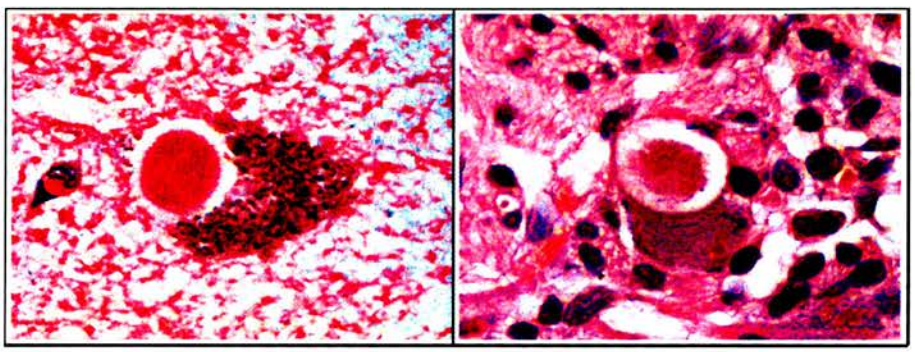


Figure 3-3. 'Lewy bodies'
A: Lewy inclusion body. Pigmented neuron substantia nigra. Parkinson's disease.
B: 'Lewy body like' cytoplasmic compaction. Cranial cervical ganglion neuron. Chronic Grass Sickness.

Cortical Lewy bodies are present in all cases of idiopathic Parkinson's disease but often only in small numbers. Larger numbers are associated with Lewy body dementia, which may be a variant of Parkinson's disease. They are poorly defined structures without an obvious halo, and may be angular. Ultrastructurally cortical Lewy bodies are composed of a felt like material of intermediate filaments associated with granular electron dense material (Lowe *et al*, 1997).

In addition, Lewy bodies have also been described in the cell processes, but only rarely the perikarya, of sympathetic ganglia of Parkinsonian and multiple system atrophy cases (Den Hartog Jager and Bethlem, 1960; Forno and Norville, 1976). Three types are described: the classical Lewy body type in the neuronal soma (rare), a second type made up of coarse, ill defined granules mixed with organelles and sometimes granulovesicular and filamentous material circling the central core and thirdly, swellings in swollen

vesicular and filamentous material circling the central core and thirdly, swellings in swollen unmyelinated cell processes. These are made up of various mixtures of dense material, filaments, membranous structures and dense core vesicles and, unlike classical Lewy bodies, are often described as having an 'inside out' appearance with the less electron dense material in the centre. Roessman *et al* (1971) interpreted these 'eosinophilic bodies' as axonal swellings in preganglionic terminals. They are reminiscent of the structures described as dystrophic axons by Gilmour (1975).

Stressed cells show high levels of expression of a set of proteins which have cell-organising and cytoprotective activities (Lowe and Mayer, 1990). They have been repeatedly associated with neurodegenerative diseases, particularly with the cytoplasmic inclusions which are pathognomonic for a number of them. Expression of these proteins should give an indication of the level of stress undergone by the chromatolytic, but otherwise often unremarkable, central neurons in grass sickness and may indicate how closely the cytoplasmic compactions are to Parkinsonian Lewy bodies.

Ubiquitin is a member of a group of proteins known as 'Heat Shock Proteins', having been discovered in cells responding to experimental heat shock. This response is an important basic cellular mechanism with enables cells to survive environmental stresses. Mutant strains of yeasts with altered ubiquitin gene expression do not withstand heat or toxic stresses that are non-lethal in normal strains (Finley *et al*, 1987) and work on a stroke model of induced transient cerebral ischemia in rats indicates that cell survival seems to depend on the degree of ubiquitin expression (Magnusson and Wieloch, 1989).

The function of ubiquitin in normal and stressed cells has been well reviewed (Lowe and Mayer, 1990; Mayer *et al*, 1991; Muller and Schwartz, 1995) Ubiquitin is a 76 amino acid basic protein which is amongst the most conserved gene products in evolution, with only one amino acid differing in the structure of ubiquitin in nematodes and in mammals. It has been shown to play an essential role in a wide variety of cellular

functions ranging from the regulation of chromatin conformation and embryonic development to programmed cell death and protein degradation.

Molecular and pathological findings indicate that ubiquitin may be pivotal in the cell stress response in chronic neurodegenerative diseases. Ubiquitin is induced in cells as part of the cell stress response and is a co-factor for ATP dependent non-lysosomal, and possibly lysosomal (Dickson *et al*, 1990), proteolysis, using a complex set of enzymes to finally covalently link ubiquitin to target proteins. These are then recognised and degraded, releasing ubiquitin and peptides. Virtually all misfolded proteins that have failed to find their partners undergo ubiquitination. Proteins tagged with four or more ubiquitin molecules are targeted for destruction (Mezey *et al*, 1998). As such ubiquitin appears to have been adapted as part of an intracellular surveillance system that can be activated by altered, damaged, or foreign proteins and organelles, and its primary function is believed to lie in the removal of these proteins in some eukaryotic cells.

The protein is also a component of filamentous inclusions, particularly those associated with intermediate filaments, in the neurons of a number of neurodegenerative diseases such as neurofibrillary tangles and granulovacuolar bodies in Alzheimer's disease as well as Pick bodies in Pick's disease, punctate inclusions in equine motor neuron disease (Kuwamura *et al*, 1994) and Lewy bodies in Parkinson's and diffuse Lewy body diseases. Furthermore, a study using ubiquitin antibodies to detect the presence of ubiquitin in a series of non-viral intracytoplasmic inclusions concluded that ubiquitin is selectively present in non-membrane-bound filamentous inclusions such as Mallory, Crooke, Lafora and amyloid bodies, as well as Rosenthal fibres and axons of giant axonal neuropathy (Manetto *et al*, 1989).

In addition to their association with inclusion bodies, ubiquitinated proteins, or the expression of a polyubiquitin gene, are also known to be substantially increased in neurons in a variety of neurodegenerative diseases including Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Heggie *et al*, 1989; Bergmann,

1993), as well as in non-filamentous 'dot like structures' in normal brains (Dickson *et al*, 1990). Ubiquitinated inclusions in CNS LMNs in ALS are distinct from conventional intermediate filament inclusions and may be highly disease specific as well as being present in transgenic mouse models (Ince *et al*, 1998).

b) Materials and methods

(i) Subjects

The initial investigation focused on sections of cranial cervical ganglia from four acute, two subacute, one chronic and two control cases. In the next phase, central neurons were examined using sections from three areas of the brain stem which included the motor nuclei of cranial nerves III, V and VII. Four sets were from cases of subacute grass sickness and five from chronic cases, all with prominently chromatolytic neurons in two or more of these nuclei on H&E evaluation. In addition, one longitudinal section of spinal cord from a case of subacute grass sickness was used, principally because it was known to contain a large number of affected lower motor neurons, many more than could be examined in one section of brain stem. One horse with colic and one orthopaedic case served as negative control cases. Each run incorporated a control positive section of human cortex with prominent corpora amylacea, and at least one presumed positive control equine section of either caudal brainstem containing the lateral cuneate nucleus from a case of equine degenerative myeloencephalopathy (EDM), or spinal cord from a severe case of cervical vertebral malformation (CVM).

(ii) Laboratory methods

Ubiquitin immunoreactivity was revealed using a standard avidin/biotin immunostaining protocol. 5 μm sections on Vectabond (Vector Laboratories, Inc., CA) coated slides were incubated in a 70° C oven for 30 minutes before being deparaffinised in xylene, rehydrated through graded alcohols and placed in picric acid for 15 minutes to decrease formalin complex background staining due to over-fixation. After rinsing in water, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water for 15 minutes. Sections were incubated in 10% normal swine serum with added avidin (Avidin/Biotin blocking kit, Vector Laboratories) for 15 minutes at room temperature in a humid chamber. Pilot studies using primary antibody dilutions from 1:50 to 1:500 determined that 1:200 rabbit anti bovine ubiquitin (Biomeda) with added biotin, preincubated in 2% normal horse serum (NHS) for 1 hour at 37°, was optimal, and this was applied and the slides incubated for 30 minutes at room temperature. 1:200 non-immune rabbit serum (NRS), preincubated in 2% normal horse serum ,was applied to cranial cervical ganglia negative control slides. Polyclonal Universal Negative Control Serum (CPD, Llanberis) but no NHS preincubation or avidin/biotin blocking step was used for negative control slides in central sections in subsequent studies. Biotinylated 1:200 swine anti rabbit immunoglobulin (DAKO) was applied as the secondary antibody for 30 minutes at room temperature in a humid chamber and slides were incubated in the ABC Reagent (StreptABComplex/HRP, DAKO) for 30 minutes at room temperature. Sections were developed by incubating in a DAB (3,4,3',4'-tetra-amino biphenyl hydrochloride) Substrate Kit (Vector Laboratories, Burlingame CA 94010) at room temperature for 30 seconds and counterstaining with hematoxylin. Finally, the slides were dehydrated in graded alcohols and three changes of xylene and mounted in Permount (Fisher Scientific).

Table 3-2: Subjects and sections examined for ubiquitin expression.

Case Number	Diagnosis	Structure of interest
96/291	SGS	Nucleus of CN III, CN XII, GVE X,
96/396	SGS	Nucleus of CN III, motor V, CN VII
97/1032	SGS	Nucleus of motor V, CN VII, GVE X, CN XII
97/424	SGS	Nucleus of CN III, motor V, CN VII
97/293	CGS	Nucleus of CN III, motor V, CN VII
97/313	CGS	Nucleus of CN III, motor V, CN VII
97/376	CGS	Nucleus of CN III, VII, GVE X, CN XII
97/538	CGS	Nucleus of CN III, motor V,
98/348	CGS	Nucleus of CN III, motor V, CN VII
95/868	AGS	CCG
96/368	AGS	CCG
96/377	AGS	CCG
96/397	AGS	CCG
96/475	SGS	CCG
97/375	SGS	CCG
97/376	CGS	CCG
Positive Control		
98/387	GME	LMNs
Aged human		Corpora amylacea
N96- 657	CVM	Spinal cord axonal spheroids
N96-879	EDM	Spinal cord axonal spheroids
N 96-542	EDM	Spinal cord axonal spheroids
N96/847	EDM	Spinal cord axonal spheroids
Negative Control		
97/352	Orthopaedic	Nucleus of CN III, motor V, CN VII
97/618	Colic	Nucleus of CN III, V, VII, XII, GVE X
97/654	Hepatopathy	CCG
96/500	CVM	Spinal cord axonal spheroids

CCG = cranial cervical ganglion

c) Results

The positive control human subependymal corpora amylacea stained dark brown in all the ubiquitin runs with no non-specific binding elsewhere. The lateral cuneate nucleus from one case of EDM contained several dark accumulations which may represent small spheroid or axons. Compressed equine spinal cord sections did not contain many prominently labelled spheroids but numerous astrocyte nuclei were labelled as well as a number of the nuclei of alpha motor neurons.

Negative control sections from both horse and human negative control sections were marked by high levels of non-specific staining when NRS was applied at the same dilution as the diluent containing the primary antibody. No evidence of specific binding of equine tissue was found using the commercial negative control product.

Numerous postganglionic sympathetic neuronal nuclei in both grass sickness and normal control cases were labelled with the rabbit anti bovine ubiquitin. Most of these had an 'open faced' morphology. The cytoplasm of many neurons was also labelled, with or without associated nuclear labelling. Degenerate neurons did not appear to be labelled preferentially over normal appearing cells in grass sickness cases. 'Lewy body like' structures, or cytoplasmic compactions to varying degrees, were occasionally labelled but not consistently and not strongly (Figure 3-4).

In the central sections in both grass sickness and control cases the ependymal cells around the mesencephalic aqueduct were often labelled, as were astrocyte nuclei. Small accumulations of pigment were occasionally seen in the neuropil, not associated with particular nuclei and not in greater quantities in grass sickness compared to control cases. The was no significant cytoplasmic staining of neurons in the cranial nerve nuclei under examination and the labelling of neuronal nuclei could not be differentiated from similar staining in normal appearing somatic neurons in grass sickness or normal control cases.

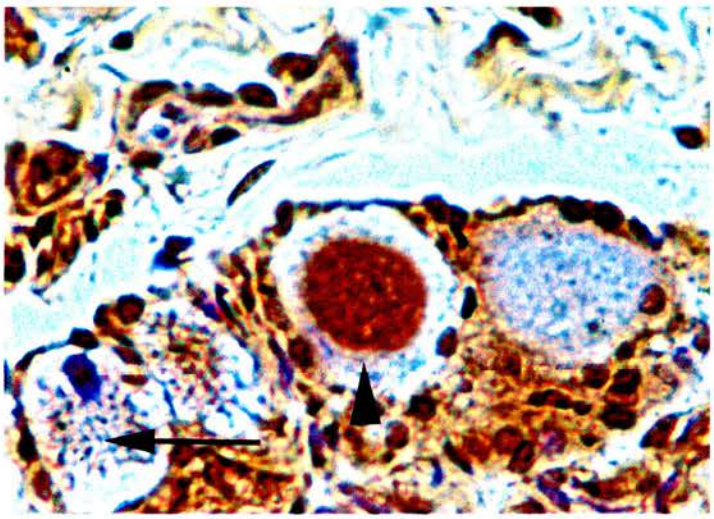


Figure 3-4: 'Lewy body like' cytoplasmic compaction labelled with ubiquitin (arrowhead) and unlabelled (arrow). Postganglionic sympathetic neurons, cranial cervical ganglion, chronic grass sickness. Ubiquitin 1:200. X 200.

d) Discussion

The results obtained were initially thought to include an un-interpretable amount of false positive and false negative labelling due to the apparent absence of staining of axonal spheroids, intense background staining of normal rabbit serum treated sections and labelling of lower motor neuron and astrocyte nuclei. The lack of a known true positive equine tissue was an additional obstacle to interpretation of the results.

The rabbit anti ubiquitin was supplied as part of a kit and the true concentration of antibody relative to the concentration of immunoglobulins in NRS was unclear and relatively high concentrations of NRS were initially applied to negative control sections leading to a an unacceptably high level of background staining. This was probably due to non-specific rabbit anti horse and anti human interactions. The Avidin / Biotin blocking and preincubation in normal horse serum steps were introduced to try to decrease non specific rabbit anti horse, and avidin by endogenous biotin binding. Further discussion with Biomeda revealed a much lower content of non-specific immunoglobulins in the

primary antibody than was initially understood, and a commercial negative control product was subsequently applied to the central section.

Standard positive control tissues for ubiquitin work include either human corpora amylacea, subpial or subependymal structures associated with ageing, or Parkinsonian Lewy bodies. Since neither of these structures were available in horse tissues, alternative structures likely to contain ubiquitinated proteins were sought. Axonal spheroids are known to express ubiquitin epitopes in a variety of neurodegenerative (Lowe and Mayer, 1990; Moretto *et al*, 1993; Takahashi *et al*, 1997) and traumatic diseases including compressed equine spinal cord (Jortner *et al*, 1996). The specific brainstem and spinal cord nuclei from cases of EDM classically contains spheroids (Mayhew *et al*, 1977) as do compressed segments of spinal cord from animals with cervical vertebral malformation and sections from these were included as positive equine control tissues.

The lack of consistent labelling of axonal spheroids, and the strong staining of many nuclei in this experiment however does not necessarily indicate non-specificity of the ubiquitin antibodies for equine tissues, something that would be unexpected given the great similarity of the ubiquitin molecule across species. Spheroids from cases of human dysautonomia show reactivity with antisera to ubiquitin only when smaller than 30 microns, and it is speculated that larger spheroids containing degradative products of neurofilament proteins may lose their immunoreactive epitopes (Moretto *et al*, 1993). The small structures labelled in the region of the lateral cuneate in the EDM tissues may represent similar smaller dystrophic axons. In addition, not all spheroids in the spinal cord segments from horses with CVM were intensely labelled with ubiquitin in one study (Jortner *et al*, 1996), but many were seen to demonstrate this protein in a more consistent and intense fashion than seen in one control animal. In addition, nuclei have been reported to be frequently labelled in human tissues expressing ubiquitin as well as occasionally in neurons, Schwann cells and glial cells of normal CNS (Manetto *et al*, 1988; Dickson *et al*, 1990).

The presence of heat shock proteins in other neurodegenerative diseases however has not been explained (Lowe and Mayer, 1990; Mayer *et al*, 1991; Mezey *et al*, 1998). A recent study looking for a number of stress proteins in the brain of Alzheimer disease patients using non-quantitative immunohistochemistry, including alpha B-Crystallin, heat shock protein (HSP) 27, HSP 65, HSP 70, HSP 90, and ubiquitin, concluded that a selective up or down regulation of stress proteins in degenerating neurons could not be detected (Stadelmann *et al*, 1998). The use of alpha B-Crystallin and heme oxygenase-1 antibodies was attempted in this study, but was abandoned as their specificities could not be determined due to the lack of positive control tissues in this species. Proteins may be ubiquitinated in an effort to degrade abnormal organelles or proteins, accumulated as part of a pathologic process, or they may actually cause the neuronal death. Alternatively they may initially be cytoprotective and, if this fails, become involved in a ubiquitin-dependent protein catabolism which ultimately destroys the neurons (Mayer *et al*, 1991).

Similarly, the role of inclusion bodies is not understood, particularly since their formation does not necessarily correlate with the expression of heat shock proteins associated with various forms of cell stress such as cerebral ischaemia. Intermediate filaments are the elements of cytoskeleton most sensitive to cell stress, and ubiquitinated intermediate filament inclusion bodies may represent a failure of protein degradation system, or may be result of an attempt to cocoon unwanted cellular material. The inclusions may then become permanent or they may be slowly eliminated by a ubiquitin coupled breakdown system.

It is likely that the argyrophilic inclusions described as dystrophic axons by Gilmour (1975) represent the perikaryal structures resembling Lewy bodies which are commonly seen in the sympathetic ganglia of subacute and chronic cases. The presence of a single limiting membrane would differentiate them from classical Parkinsonian Lewy bodies, which are not surrounded by a membrane (Lowe *et al*, 1997). The structures are only variably argyrophilic in our laboratory (Figure 3-5), however that may be dependent on

described apparently extracellular Lewy bodies in the Parkinsonian autonomic nervous system. He debated that these are likely to represent intracytoplasmic Lewy bodies protruding slightly from the cytoplasm and appearing to be extracellular in some sections, or a very large intracytoplasmic Lewy body filling the entire cell which finally degenerates, giving rise to an extracellular body. Alternatively, they may come from degenerated dendrites or neurites cut in transverse sections. The former two options may be more likely based on the fact that some of these structures in grass sickness appear to be placed firmly in the perikaryon, which would not be compatible with their description as dystrophic axons. It is possible, if somewhat unlikely, that these structure are present in both soma and axons. Closer examination of postganglionic sympathetic axonal morphology may determine if this is the case.

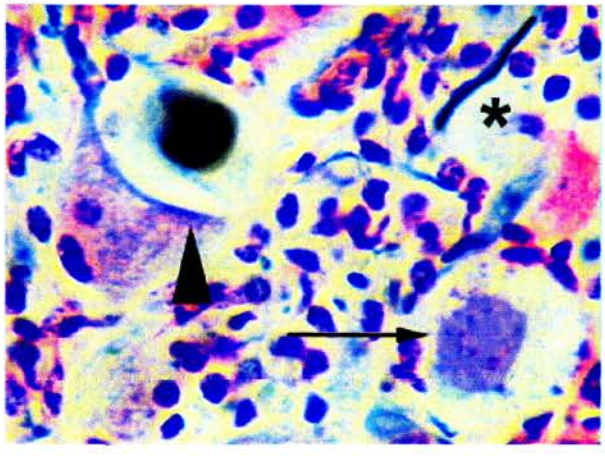


Figure 3-5: Argyrophilic (arrowhead) and unstained (arrow) cytoplasmic compactions. Labelled axon (star). Cranial cervical ganglion, Holmes Silver, x 200.

e) Conclusion

Ubiquitinated inclusion bodies are not a feature of Grass Sickness nor does there appear to be a substantial increase in ubiquitin expression. More sensitive techniques, such as

e) Conclusion

Ubiquitinated inclusion bodies are not a feature of Grass Sickness nor does there appear to be a substantial increase in ubiquitin expression. More sensitive techniques, such as quantitative in-situ hybridisation methods for evidence of differences in RNA expression, may disclose subtle increases in expression of this heat shock protein in chromatolytic central neurons from cases of EGS.

4. NEUROFILAMENTS

a) Introduction and literature review

Neurons contain three major classes of cytoskeletal elements, microtubules, microfilaments and neurofilaments, interconnected by protein bridges. Neurofilaments are neuron-specific, stable, intermediate filaments and have been recognised for over 100 years due to their intense argyrophilia. The three protein subunits, light (L), medium (M) and heavy (H), are assembled in the perikaryon and move down the axon, where they fill most of the axoplasm, until they are degraded at the axon terminals. Their function is still conjectural, but may contribute to the stability and rigidity of the axon (Greenfield *et al*, 1988). Unlike in the soma, neurofilaments in axons are phosphorylated. Phosphorylation of neurofilaments begins in the cell body of central neurons but increases markedly in the axon once the neurofilaments begin to be transported (Manetto *et al*, 1988).

Soma which are chromatolytic secondary to axonal damage, accumulate phosphorylated neurofilaments associated with the decrease in their outflow from the cell body into the axon and the longer residence time in that region of the neuron (Manetto *et al*, 1988). Alterations in the neuronal cytoskeletal architecture are one of the most generalised neuropathologic changes in neurodegenerative disorders (Nicotera *et al*, 1997) and the perikaryon is known to accumulate phosphorylated neurofilaments of a number of neurodegenerative diseases including motor neuron disease in man (Munoz *et al*, 1988; Sobue *et al*, 1990; Gonatas *et al*, 1992; Lowe *et al*, 1992; Takahashi *et al*, 1997; Ince *et al*, 1998), Brittany spaniels (Cork *et al*, 1988; Price *et al*, 1996), rabbits and pigs (Cork *et al*, 1988), cats (Shelton *et al*, 1998), and mice (Koliatsos *et al*, 1994), as well as in other neurodegenerative diseases such as Werdnig-Hoffmann's disease, multiple system atrophy (Sobue *et al*, 1990) and neuronal inclusions on Parkinson's and Alzheimer's diseases (Manetto *et al*, 1988). It has been proposed that phosphorylation of NF in ALS

occurs prematurely and is associated with an impairment of neurofilament transport (Manetto *et al*, 1988).

Griffiths (1993) recorded marked disturbances in neurofilament proteins in postganglionic sympathetic neurons in EGS. The purpose of the present study is to determine if phosphorylated neurofilaments accumulate in the perikaryon of central neurons in EGS.

b) Method

(i) Subjects

24 sections of tissue containing the nucli of CN III, V, VII, XII or GVE X from10 EGS cases and three control animals were included.

(ii) Laboratory methods

Immunohistochemistry was performed by the peroxidase-anti peroxidase method.

Five μm sections on Vectabond (Vector Laboratories, Inc) coated slides were incubated in a 70° C oven for 30 minutes before being deparaffinised in xylene, rehydrated 100% alcohol and placed in Lugol's iodine followed by 5% sodium thiosulphate for one minute to clear preservative precipitates and enhance staining. After rinsing in water, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in absolute alcohol 15 minutes. Sections were rinsed in PBS. Sections were incubated in 10% normal goat serum for two hours at room temperature in a humid chamber. Mouse monoclonal anti-phosphorylated neurofilament H and M antibody (1:1500 SMI 31, Affinity, Research Products Ltd) was applied overnight at 4°C. PBS was applied to a negative control section in each run. Goat anti-Mouse IgG (Sigma Immuno Chemicals,

(BDH Laboratory supplies, UK), incubated for five minutes, counterstained with hematoxylin for 20 seconds and mounted.

c) Results

Axons and an occasional incidental spheroid were strongly positive in tissues from both EGS cases and control animals. A few soma on sections from control animals had very light stippled staining but none were strongly labelled. A number of GSE and GVE neurons (usually ~20%) showed intense cytoplasmic labelling of the soma (Figure3-6). A proportion of them appeared to be chromatolytic, with a swollen soma and central staining, but the majority were morphologically normal with diffuse labelling of the cytoplasm.

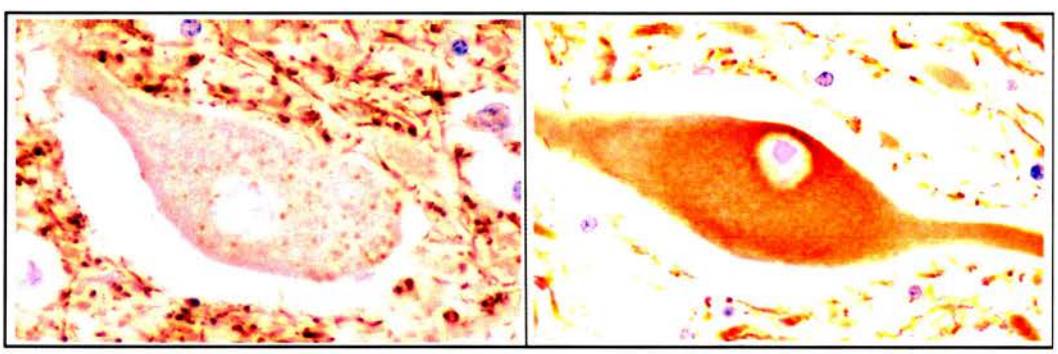


Figure 3-6: Labelling of control (A) and EGS (B) neuronal soma. SMI 31 1:1500

d) Discussion

The labelling of phosphorylated neurofilaments in the soma of central neurons is not found in normal tissues. This is unlikely to be due to primary axonal disease since, as presented in chapter 4, the peripheral nerve from nuclei with chromatolytic neurons do not show evince of pathology. This suggests that premature phosphorylation is taking place, which may be either the result or the cause of an axonal transport problem. Semi-quantitative work in ALS has shown a significant increase in the phosphorylation of neurofilaments not only in the perikaryon but also in the proximal region of the axon with an accompanying decrease in labelling of non-phosphorylated neurofilaments. These data suggest that phosphorylation of neurofilaments occurs prematurely in the cell body and proximal axons of patients with ALS thereby masking the non-phosphorylated epitopes. It may be possible to differentiate a generalised neurofilament transport problem in EGS from an unrelated defect resulting in inappropriate premature phosphorylation, by assessing whether there is an accumulation of non-phosphorylated neurofilaments as well. Immunohistochemical staining for non-phosphorylated neurofilaments as well as acetylated and β tubulin was attempted in order to further define a disorder in cytoskeletal structure, however the results were not conclusive, potentially because of a lack of staining specificity.

The immunoreactivity of epitopes of phosphorylated neurofilaments in normal appearing perikaryons poses the question of whether their presence in the soma precedes the dissolution of the Nissl substance and chromatolysis, or is found in neurons that are recovering. The absence of evidence of central neuronal cell death suggests that the latter is possible. The proportion of chromatolytic neurons in all affected GSE and GVE LMNs is 0.16 (Chapter 2), which is similar to the proportion of soma labelled for phosphorylated neurofilaments in the majority of sections examined. Cases were noted to have chromatolytic neurons over a large range of disease duration (1 – 76 days), and it may well be that the process is dynamic, with some cells recovering and others

exhibiting an increase in metabolic activity and chromatolysis. In the absence of an *in-vitro* model however this question may be very difficult to answer.

These results, in addition to the work done by Griffiths (1993), point to an alteration of the neuronal cytoskeleton being part of the cytopathology of EGS. Further detailed studies may implicate a group of agents responsible.

5. ELECTRON MICROSCOPY

a) Introduction and literature review

Electron microscopic examination of material from EGS cases was initially performed by Chandler (1967), focusing on intestinal sympathetic ganglia from three affected horses. Considerable time had elapsed in some cases before fixation. The cytoplasm in all neurons examined was abnormal with gross vacuolation being a prominent feature. Vacuoles were up to 4 μm in diameter, and were limited by a single, often incomplete membrane with occasional fusion of adjacent vacuoles and a very low density content. One case was marked by large numbers of dense structures, 0.25 – 0.3 μm in diameter, with a circular profile, a double membrane and with a variable content. It was speculated that these structures represented stages in development of the vacuolation. In addition, altered mitochondria were observed, however the state of preservation must hinder interpretation of subtle changes.

Gilmour (1975) was able to remove the cervicothoracic and middle cervical ganglion within five minutes of euthanasia and noted nuclear pyknosis, cytoplasmic vacuolation originating from granular endoplasmic reticulum and axonal dystrophy. There was an apparent increase in microtubules but no normal rough endoplasmic reticulum was evident. Mitochondria were similar to those seen on control grids. Round or oval structures surrounded by a single limiting membrane and containing mitochondria, membrane bound vacuoles and vesiculotubular material, were noted in the recesses of neurons and in the satellite cell network (see chapter 3, section 4).

(Hodson *et al*, 1984c, Hodson, *et* al, 1997) observed marginated Nissl, crenated and peripheral nuclei, dense bodies in vacuolated cytoplasm, and an increase in lysosomes in neurons of the coeliacomesenteric ganglion. Unmyelinated axons within the ganglion were observed to contain accumulations of vesicular material, neurofilaments and mitochondria indicative of a disruption of axoplasmic flow. Some myelinated fibres showed abnormalities of the myelin sheath in terms of lamellar stripping of myelin and

vesiculation. Accumulations of dense bodies in Schwann cells were often seen, together with dilated cyternae. Similar changes were also apparent in satellite cells. Dorsal root ganglia were also examined and contained neurons which showed changes compatible with classical chromatolysis. Overall the changes were suggested to be similar to retrograde lesions following axonal pathology.

The ultrastructural appearance of central neurons in EGS have not been examined.

b) Materials and methods

(i) Subjects

Six horses with chronic grass sickness were euthanised with a mixture of quinalbarbitone and cinchocaine (Somulose, Arnolds). The head was disarticulated and the brain collected by sawing through the calvarium with an electric saw (PFZ 550 E, Bosh). The smallest sections of tissue which could reasonably be cut from unfixed brain (averaging 3 mm^3), were removed from the caudal medulla oblongata just lateral to the floor of the fourth ventricle. The average time take from euthanasia to immersion of the tissues in fixative was just under 12 minutes.

(ii) Tissues

Tissues were processed by the Electron Microscope Unit, R(D)SVS. The specimens were fixed in 3% glutaraldehyde in 0.1% sodium cacodylate buffer solution at pH 7.3 for at least two hours at room temperature. After several washings with cacodylate buffer solution, tissues were post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer solution at pH 7.3 for one hour at room temperature. Following dehydration in a graded series of acetone solutions, the tissue was infiltrated and embedded in an Araldite resin mixture. In order to orientate and select an area for the ultrastructural observations, semi-

thin 1 μm sections were cut with glass knives, stained with 1% toluidine blue and examined under the light microscope. Ultra-thin sections with silver gold interference colour (approximately 70-80 nm in thickness) were cut on an OMU4-Reichert Ultracut microtome and collected on 200 mesh grids. The sections were double stained in saturated uranyl acetate in 50% methanol for 30 minutes followed by lead citrate in water for 5 minutes. The areas selected for investigation included large neurons which were expected to originate from the hypoglossal nucleus. The grids were examined and photographed using a Philips CM 12 transmission electron microscope.

c) Results

The state of preservation was reasonable, as judged by the structure of central myelin sheaths in which the dense lines and intraperiod lines could be differentiated (Figure 3-7) and mitochondria, in which cristae could be identified (Figure 3-8).

The perikarya of normal appearing neurons were large and round or multipolar (Figure 3-9), with organelles and multiple dark lipofuscin granules containing a peripherally located vacuole dispersed throughout the cytoplasm (Figure 3-10. Nuclei were prominent and round, containing a large nucleolus and little heterochromatin. The Nissl bodies consisted of discrete stacks of orderly arrays of nearly parallel, broad cisternae with ribosomal rosettes attached to their outer surface and free clusters of ribosomes in the surrounding cytoplasmic matrix. The Golgi complex was infrequently observed, but when present it appeared normal.

Very few affected neurons were evident and only one was noted which included a nucleus. The cytoplasm of abnormal neurons were characterised by an extensive loss of membrane associated ribosomes the apparent replacement of stacks of Nissl substance by finely granular material. Soma were large and round (Figure 3-11), with numerous

mitochondria and lipofuscin granules distributed throughout the cytoplasm. The mitochondria appeared increased in number, but due to the paucity of affected neurons available this could not be substantiated. In one cell in which the nucleus was in the plane of section, the nucleus were undergoing early karyorrhexis with a partial loss of the nuclear membrane and clumping of heterochromatin (Figure 3-12). There was no evidence of neurofilamentous accumulations or cytoplasmic inclusion bodies.

Figure 3-7: Transmission electron micrograph, central myelin sheath X 160,000. DL – major dense line. IL– intraperiod line

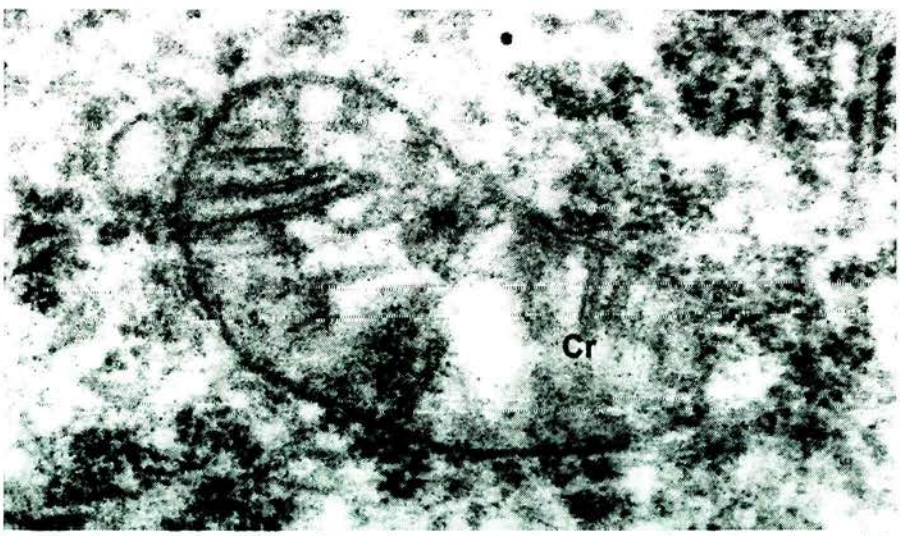


Figure 3-8: Transmission electron micrograph, mitochondrion X 45,000. Cr – cristae.

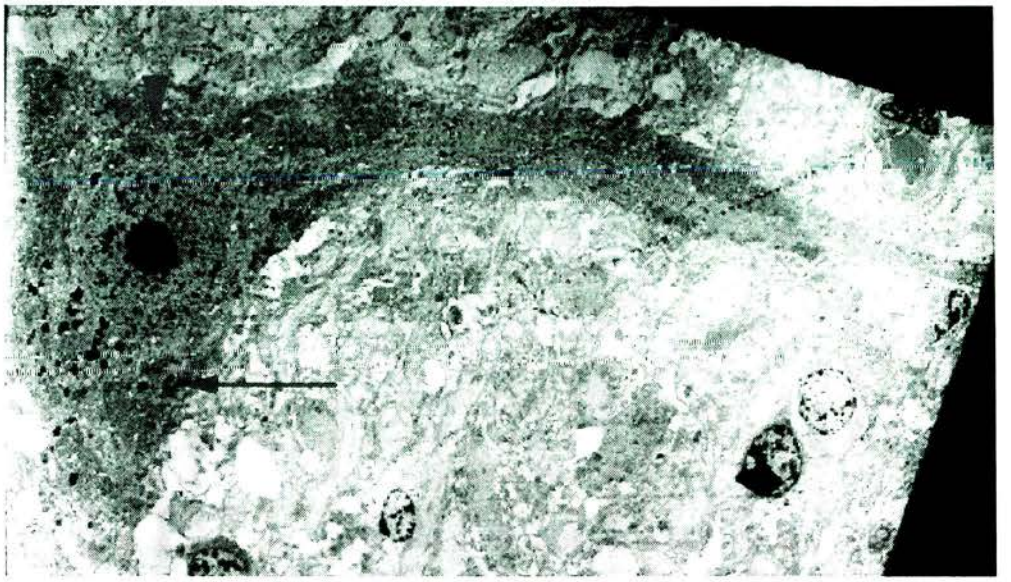


Figure 3-9: Transmission electron micrograph, normal neuron containing an open faced nucleus (arrow head) and multiple lipofuscin granules (arrow) X 1000

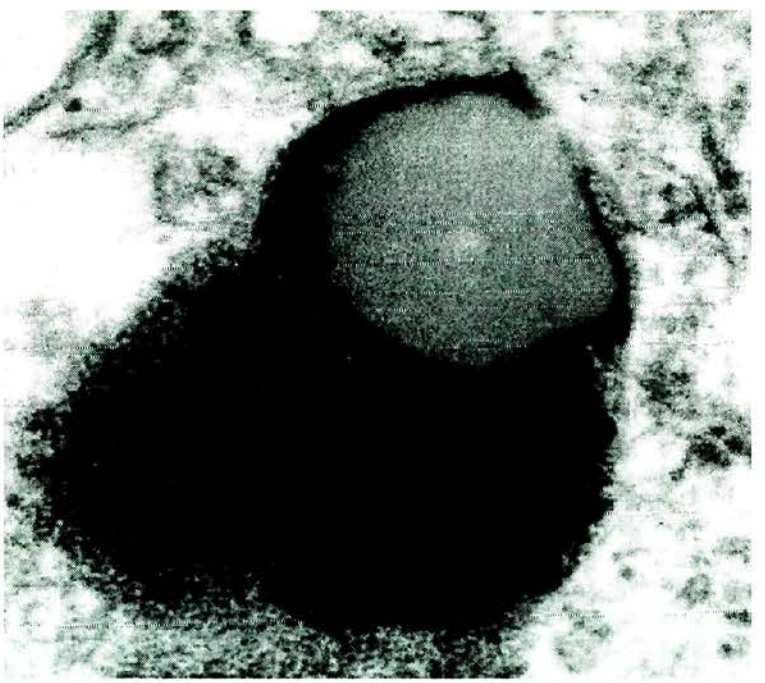


Figure 3-10: Transmission electron micrograph, lipofuscin granule X 45,000

Figure 3-11: Transmission electron micrograph of a chromatolytic neuron, CN XII, CGS. X 1000

CN XII, CGS.X 1000

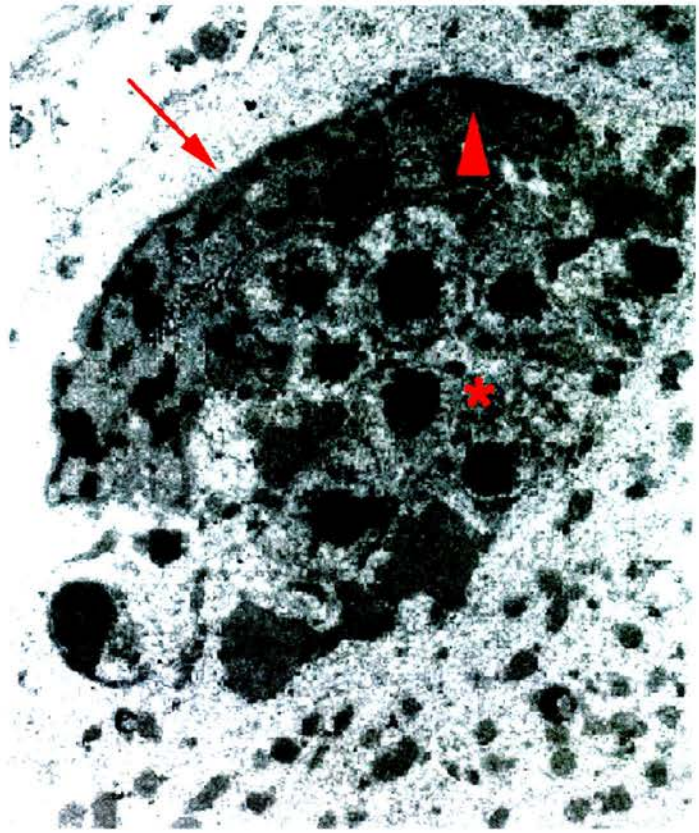


Figure 3-12: Transmission electron micrograph of the degenerating nucleus of a chromatolytic neuron showing condensed chromatin (arrowhead), a partial nuclear membrane (arrow) and nuclear fragments (star). CN XII. X 10,000

d) Discussion

The nucleus of CN XII was selected to be examined since almost 90% of horses with chronic grass sickness were noted to have chromatolytic neurons in that nucleus, and it is relatively easy to isolate. Nevertheless only very few chromatolytic neurons were successfully identified. In part, problems arose because of the difficulty involved in accurately trimming fresh brain, even when exceptionally sharp instruments were employed. The lack of *in-situ* perfusion also invariably resulted in the creation of post-mortem artefacts, despite the active measures taken to decrease the time from death to tissue collection. The brain of smaller animals is classically fixed by cannulating the aorta and perfusing with large volumes of aldehyde fixatives but the technical difficulties involved in performing this task in an animal the size of a horse however are almost prohibitive (S. Hackett and J. Cummings, Cornell University, pers comm).

The ultrastructural changes associated with chromatolysis due to axon pathology in central neurons have been well defined (Aldskogius, 1978; Thomas *et al*, 1993). Depending on the duration and distance from the axon damage and the functional system the neurons originate from, cytoplasmic changes have been noted to include the disappearance of granular endoplasmic reticulum, proliferation of smooth endoplasmic reticulum, focal mitochondrial aggregates and mitochondrial degeneration and increased numbers of microtubules and neurofilaments. This is associated with nuclear displacement, infoldings of the nuclear membrane, nucleolar condensation and vacuolation and the appearance of intranuclear electron dense particles. The findings have been interpreted as indications of disturbed protein metabolism, oxidative metabolism and increased intraneuronal transport.

The hallmark of chromatolysis, dispersal of the Nissl substance, was the principal criterion by which abnormal neurons were recognised. There was no evidence of cytoplasmic inclusions or increased neurofilaments, in direct contrast to EMND (Cummings *et al*, 1990). Mitochondria appeared relatively well preserved and not

overtly degenerating but were not objectively quantified. The state of fixation, although reasonable, did not allow for pragmatic statements to be made about the appearance of other organelles such as lysosomes, autophagic vacuoles, or of further cytoskeletal elements such as microtubules.

The accumulation of lipofuscin granules seen here is a common feature of ageing, with a mean of 63% of anterior horn neurons containing lipofuscin in 91 year old humans compared to 24% at age 17 (Thomas *et al*, 1993). Histochemically lipofuscin is heterogeneous and consists of lipids, proteins and carbohydrates, and ultrastructurally it is composed of membrane-bound particles which contain mainly electron-dense homogenous material with a granular appearance. Increased levels of lipofuscin are found in diseases associated with alpha-tocopherol deficiency, including equine degenerative myeloencephalopathy (Mayhew *et al*, 1978) and EMND, in which prominent accumulations of endothelial lipopigment are found in the small vessels of the spinal cord (Cummings *et al*, 1995) and retina (Riis *et al*, 1999). A large body of evidence suggests that lipofuscin can be used as a marker of oxidative stress and ageing (Sohal and Brunk, 1989) but lipofuscin levels have not been shown to increase in ALS patients relative to control tissues (McHolm *et al*, 1984). Barlow (1969) noted lipofuscin granules in EGS central tissues, however EGS cases are unlikely to be deficient in alpha-tocopherol and lipopigment accumulations have not been noted in the CNS vascular endothelium

The ultrastructural changes of central neurons in the nucleus of CN XII have been described in cats with dysautonomia using *in-situ* fixation of the brain (Pollin and Griffiths, 1987). Abnormal neurons were characterised by a dispersion of the Nissl substance progressing to dilation of individual cisternae by an electron-dense floccular material with those cisternae having lost the majority of their ribosomes. There was proliferation of smooth endoplasmic reticulum in several neurones with an increased number of morphologically normal mitochondria and lysosomes. The nuclei of affected

neurones were eccentric with crenations of the nuclear envelope and in some cases nucleolar changes. Neurons which appeared to be degenerating were also identified, and these contained only smooth membranous profiles and vacuoles with occasional lysosomes and mitochondria. Adjacent GVE X neurons were examined and in addition to the changes noted in the somatic neurons these contained frequent, complex, membranous stacks. It was not clear whether this is due to a difference in the severity of the initial damage to centrally and peripherally situated neurons, or whether there is a differing response to a similar insult in the two cell types.

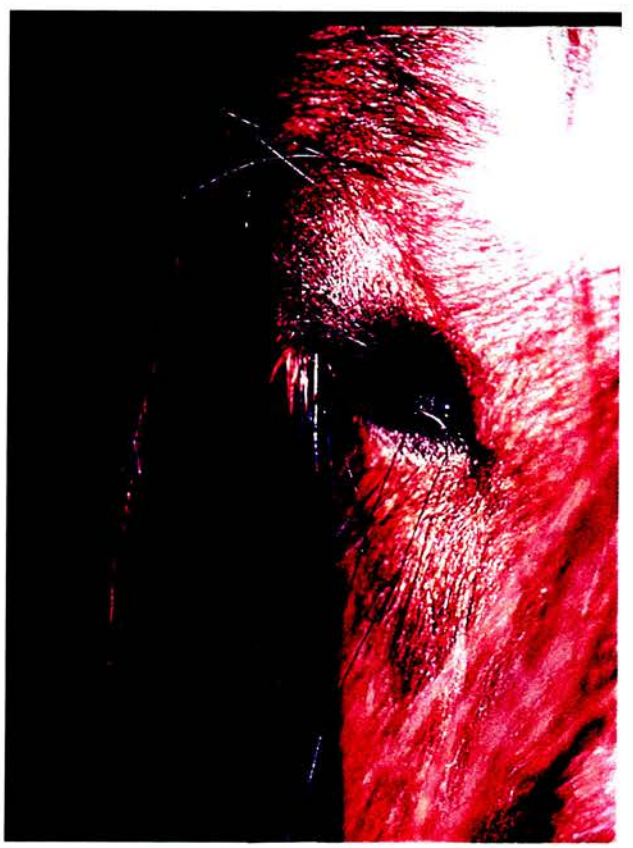
Notably, no Golgi complexes, either normal or abnormal, were identified in any central neuron in feline dysautonomia in which the Nissl substance was markedly disrupted. The Golgi apparatus was similarly not recognisable in an ultrastructural study of postganglionic sympathetic neurons in affected hares (Griffiths and Whitwell, 1993). The Golgi complex is involved in important functions of processing and transport of plasma membrane, lysosomal, and secreted proteins. Fragmentation and atrophy of the Golgi apparatus of motor neurons has been detected with organelle specific antibodies in approximately 30% of motor neurons in five ALS patients in one study (Gonatas *et al*, 1992), which suggests that the alteration of the Golgi apparatus is an early event in the pathogenesis of the neuronal degeneration in ALS. The Golgi apparatus was not evident in affected neurons in the present study, however it would be preferable to be able to examine further affected central neurons in EGS, particularly if *in-vivo* fixation could be employed.

In summary, the ultrastructure of chromatolytic central neurons of EGS cases indicates that these neurons have a dispersed Nissl substance, no evidence of neuronal inclusions and an apparent loss of Golgi apparatus. In contrast to evidence found on histopathology and TUNEL staining, which indicates that there is no evidence of neuronal cell death in central tissues from EGS cases, the presence of at least one pyknotic nucleus examined suggests that some affected neurons may not be capable of recovering. Indeed, the

presence of karyorrhexis in at least one nucleus suggests that apoptosis may after all be occurring in central neurons from EGS cases.

Chapter Four

Ptosis



1. INTRODUCTION TO PTOSIS IN EGS

Ptosis in EGS could reasonably be caused by combinations of pathology to the structures that assist raising the upper eyelid (Figure 4-1). The principal muscle elevating the upper eyelid under voluntary control is the *levator palpebrae superioris*, a flat muscle situated almost entirely in the orbit, originating on the pterygoid crest and terminating in a thin tendon in the upper lid. It is innervated by CN III. Ptosis due to pathology to CN III is rarely seen in isolation, but can be associated with focal pathology caused by retrobulbar masses, or more diffuse diseases including polyneuritis equi (Mayhew, 1989).

An additional skeletal muscle is the *levator anguli oculi medialis* (LAOM), a small muscle lying on the frontal bone and inserting on the medial part of the upper lid (Budras *et al*, 1994). The muscle is innervated by a branch of CN VII, the auriculopalpebral nerve, and unlike in dogs (Braund, 1994), or in man (Adams and Victor, 1993), facial nerve paresis in horses does result in ptosis (de Lahunta, 1986), as that muscle does not appear to play a role in raising the eyelid of species other than horses or cattle.

Resting tone of the eyelid is supplied by a smooth muscle, Müller's superior tarsal muscle underlying the striated levator muscles of the eyelid. It is innervated by postganglionic sympathetic axons from neurons in the cranial cervical ganglion. In the dog this is a delicate fan-shaped muscle arising from the trochlear cartilage on the medial wall of the orbit, inserting on the tarsus in the upper lid continuous with the edge of the *levator palpebrae superioris* muscle (Evans, 1993). Müller's muscle has also been referred to in the lower eyelid of the dog but does not appear to have been described in either eyelid in the horse. In man the upper lid becomes ptotic by no more than 2 to 3 mm when cervical sympathetic nerves are interrupted (Beard, 1983) but in horses ptosis is one of the most constant and distinct features of Horner's syndrome (Simoens *et al*, 1990). Since enophthalmos is not a feature of Horner's syndrome in horses (Simoens *et al,* 1990), ptosis due to sympathetic denervation in this species is likely to result from paresis of Müller's muscle.

Ptosis in cases of EGS could reasonably be to due to paresis of the striated *levator palpebrae superioris* or the *levator anguli oculi medialis* following dysfunction of the chromatolytic neurons of CN III or VII (Chapter 2), or due to a of the loss of sympathetic tone in Müller's muscle secondary to the pathology of neurons in the cranial cervical ganglion. Assessing the structure and function of the individual anatomical components is necessary to answer that question, and is the basis of this chapter.

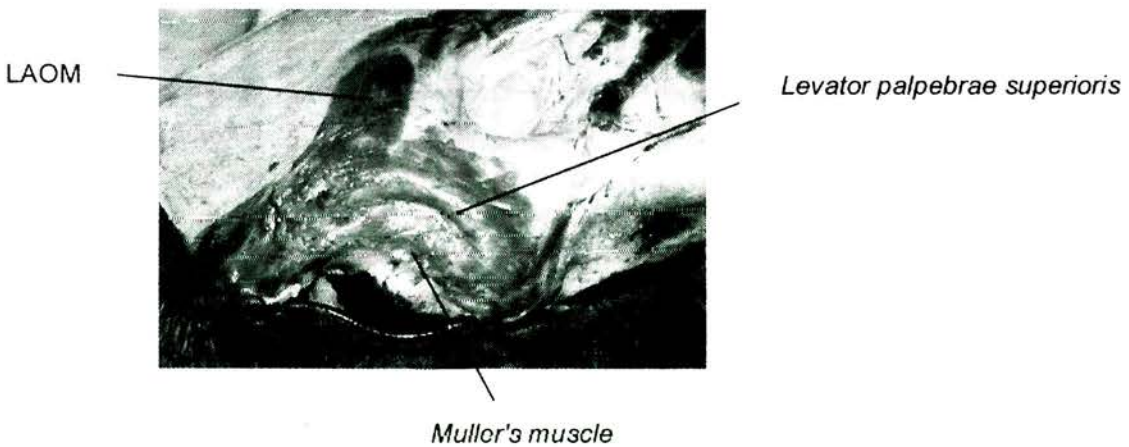


Figure 4-1: Periorbital region of the horse showing the position of muscles raising upper eyelid. Left dorsolateral view.
LAOM = Levator anguli oculi medialis

2. STUDIES ON THE EXPERIMENTAL INDUCTION OF PTOSIS IN HORSES

a) Introduction

Ptosis is a relatively common clinical sign of a variety of neurological diseases. In horses it has been noted in such diverse conditions as guttural pouch mycosis, equine protozoal myelonencephalitis, neck trauma, grass sickness, otitis media/interna, cranial nerve (CN) V pathology with marked temporal and masseter muscle atrophy, head trauma and fractures of the temporohyoid bone, among others (Smith and Mayhew, 1977; de Lahunta, 1983; Mayhew, 1989; Simoens *et al*, 1990; Cottrell *et al*, 1999).

Earlier observations in these studies indicated that assessing the angle of the eyelashes to the cornea was an effective way of determining the degree of ptosis in equine cases of Horner's syndrome. This is likely to be due to a smooth muscle, not Muller's muscle, which is mentioned rarely in the literature, the *arrectores ciliorum*, bundles of smooth muscles which extend from the follicles of the eyelashes towards the cartilaginous tarsal plate. These are absent in man but common in ruminants (Gelatt, 1991) and horses (R.E. Habel, Cornell University, personal communication). In these experiments the change in eyelash angles was used as a measure of change in the degree of ptosis.

The precise appearance of ptosis in horses due to lesions at different sites has not been documented, and was the basis of this study.

a) Materials and Methods

Six mature, healthy research ponies were used in this study (PIL 60/5641, PPL 60/1875). Five separate investigations, detailed below, were performed over three days in a random order. A minimum of five hours was scheduled between experiments to allow all clinical signs to recede.

The experiments were based on assessing the degree of ptosis by measuring the angle between the eyelashes and a line drawn horizontally between the medial canthi of the eyes (Figure 4-2). One eye was directly treated in each experiment with the untreated eye serving as a control. The relative degree of ptosis was assessed by calculating the difference in eyelash angles between the control eye and experimental the eye. Thus a positive eyelash angle difference (Diff) indicated a greater relative degree of ptosis in the experimental eye (Tx), whereas a negative eyelash angle difference indicated a greater relative degree of ptosis in the control eye. In addition, a subjective visual score of ptosis was made (0 = none, 1 = just evident, 2 = mild, 3 = moderate, 4 = pronounced).

1. Topical alpha-adrenergic agonists

Phenylephrine (500µl, 0.5%w/v Martindale) was applied to the conjunctiva of the left or the right eye. Frontal photographs were taken 30 minutes later with a 1.4 M pixel digital camera (Olympus D-600L) and the subjective ptosis score was noted. Images were downloaded onto a computer and the angles of the eyelashes of both eyes determined (Image-Pro Plus, Media Cybernetics), (Fig. 4-2).

2. Topical alpha-adrenergic agonists post sedation

Within five minutes of sedation with either 500 µg/kg romifidine (Sedivet, Boehringer Ingelheim) IV, 0.5mg/kg xylazine (Virbaxyl 10%, Virbac Ltd) IV or 50µg/kg acepromazine (ACP injection, C-Vet) IV, phenylephrine was applied to the conjunctiva of one eye . The degree of sedation was noted and frontal photographs were taken 30 minutes later and analysed as above for eyelash angle difference and ptosis score.

3. Vagosympathetic trunk anaesthesia

i) The jugular vein was digitally compressed in the cranial neck and a 40 mm 21G needle inserted to its hub into the jugular groove in the caudal third of the neck and positioned so that blood could not be aspirated. Ten ml of mepivacaine (Intra-Epicaine, Arnolds), with or without 5ml bupivacaine hydrochloride (Marcain, ASTRA Pharmaceuticals Ltd) was injected and the onset and character of clinical signs noted. In three cases, (ponies 2, 4 and horse 6), a further 5 or 10 ml mepivacaine was administered when no facial sweating or ptosis was noted within 30 minutes of initial administration. Frontal and lateral photographs were obtained when clinical signs appeared most prominent, variably within 30 to 45 minutes of administration of the last doses of local anaesthetic. Ptosis score and eyelash angles were recorded.

ii) 5 ml of 0.5%w/v phenylephrine eyedrops were applied into one eye when ptosis appeared most prominent and the ptosis score and eyelash angles was again recorded at the time of any prominent change in eyelash angles, usually 30 to 45 minutes later.

4. Palpebral nerve anaesthesia

i) 500 µl of mepivacaine were infiltrated around the palpebral nerve at the level of the zygomatic arch using a 16 mm 23 G needle and details of the onset and character of the subsequent clinical signs were noted. A frontal photograph was taken when clinical signs appeared very prominent and the ptosis score as well as the angle of the eyelashes to a line drawn between the medial canthi of the eyes was measured as before.

ii) 500 µl of 0.5%w/v phenylephrine eyedrops were then applied and eyelids assessed as above 30 minutes later.

5. Topical adrenergic antagonist

A volume of 500 µl of 0.5 % dapiprazole monohydrochloride (Glamidolongelini, Angelini) was applied to the conjunctiva of one eye. The eyelids were assessed as above when clinical signs appeared most prominent, usually within 30 minutes of application.

Data were analysed using a statistical software package (SigmaStat, SPSS Science).

b) RESULTS

The eyelash angles determined for the treated eye, the control eye, and the difference between them, as well as the ptosis score and further clinical observations for each experiment are given in Tables 4-1 to 4-7.

The combined data were normally distributed but failed the equal variance test. A Kruskal-Wallis one way analysis of variance on ranks was performed on the difference in eyelash angles and a statistically significant ($p = 0.045$) difference among experiments was detected. Data from individual experiments passed the equal variance test and a student's t-test between the difference in eyelash angles before and after phenylephrine treatments following vagosympathetic trunk anaesthesia showed a significant difference ($p = 0.03$), indicating that the ocular sympathetic agonist had substantially reversed the degree of ptosis. Eyelash angle differences, pre and post phenylephrine administration, from animals with a palpebral nerve blockade however were not significantly different from each other ($p = 0.95$), nor were the data between phenylephrine treated and control sides in the absence of Horner's syndrome or facial paresis (experiment 1). Summary statistics and p values for student's t-test comparing experimental and control eye data for all experiments are given in Table 4-8 and Figure 4-3.

The degree of sedation was scored as 'none' or 'mild' after acepromazine or xylazine administration, and 'marked' to 'profound' after detomidine or romifidine sedation.

There was no correlation ($p = 0.68$) between the ptosis score or eyelash angle difference and the degree of sedation (experiment 2). The effect of topical adrenergic agonists and antagonists on the pupil was assessed subjectively and no conspicuous changes in diameter were noted.

There was a significant relationship between the differences in eyelash angles and the ptosis score (Spearman Rank Order correlation, $p < 0.0001$) for all experiments, however the correlation was less significant ($p = 0.02$) when only the palpebral nerve block (experiment 4i) was analysed.

Table 4-1: Experiment (1) Topical phenylephrine

	Eyelash angles:				Notes
	Control	**Rx**	**Diff**	**Score**	
Pony 1	150	146	4	0	No pupil changes
Pony 2	140	133	7	0	No response at 30 minutes
Pony 3	112	118	-6	1	No pupil changes
Pony 4	128	142	-15	3	No pupil changes
Pony 5	152	154	-2	0	No pupil changes
Horse 6	119	118	1	0	No pupil changes

Table 4-2: Experiment (2) Topical phenylephrine post sedation

		Eyelash angles:				Degree of sedation
	Sedative	**Control**	**Rx**	**Diff**	**Score**	
Pony 1	Xyl	117	155	-38	3	Mild
Pony 2	Rom	129	147	-19	4	Marked
Pony 4	Det	112	160	-47	4	Marked
Pony 5	Det	122	137	-15	3	Profound
Horse 6	Det	107	124	-17	2	Profound
Pony 3	ACP	103	101	2	0	Mild
Pony 4	ACP	114	127	-13	3	None
Pony 5	ACP	114	127	-13	3	None
Horse 6	ACP	107	109	2	0	Mild

Xylazine (Xyl), detomidine (Det), romifidine (Ram), acepromazine (ACP).

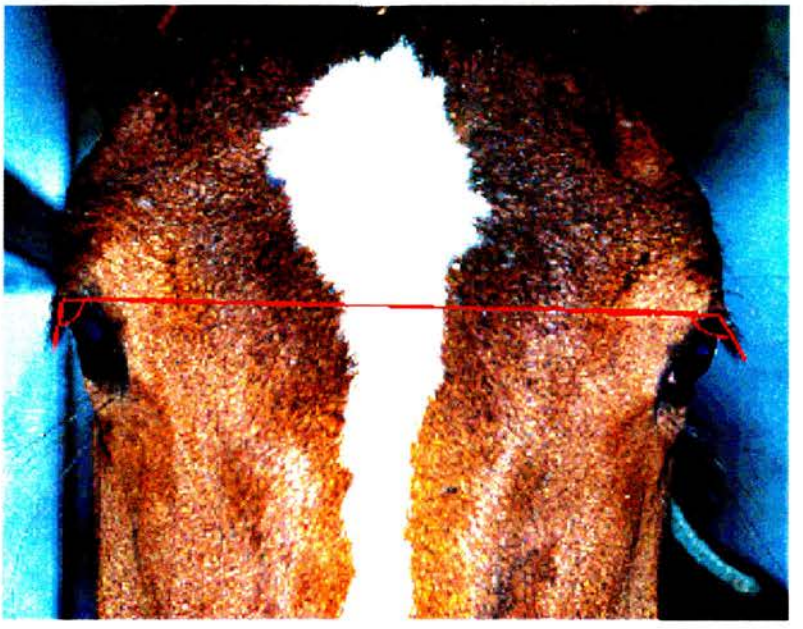


Fig 4-2. Frontal photograph of a horse with Horner's syndrome in the right eye with position of defining lines used to measure the eyelash angles

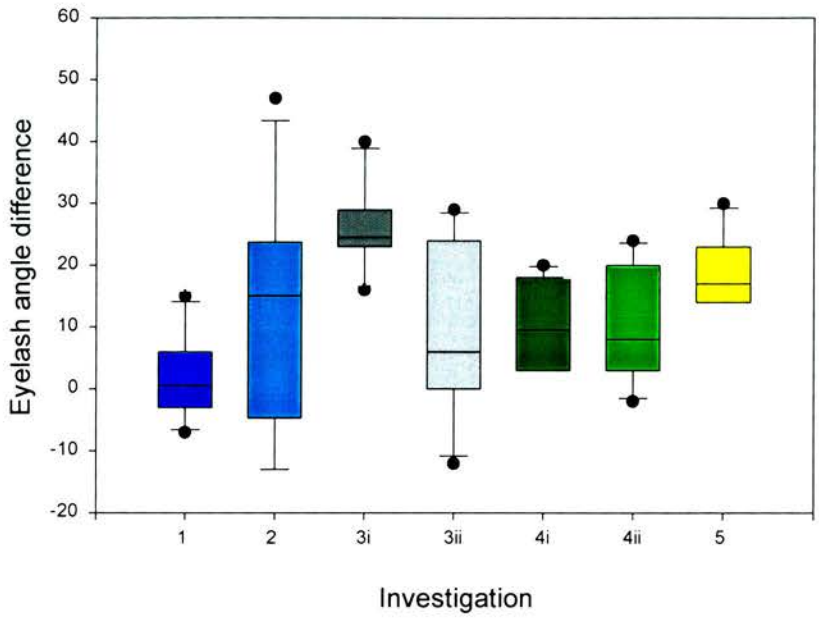


Fig 4-3. Box and whisker plot summarising the eyelash angle differences data for each investigation
Key: The ends of the boxes define the 25th and 75th percentiles with a line at the median and error bars defining the 10th and 90th percentiles for experiments A through E. Outliers are shown as •

c) DISCUSSION

The measurement of eyelash angles from a frontal view is a novel approach for assessing ptosis in equidae and circumvents the problem of simultaneously assessing the palpebral fissure of both eyes. The eyelash angle differences correlated well with the subjective ptosis score but the association was less marked when the ptosis was due to somatic CN VII nerve blockade. This was predictable given that the *arrectores ciliorum* is a smooth muscle inserting on the eyelashes. For the same reason, phenylephrine did not have a significant effect on the palpebrae of eyes in which there had been no compromise to sympathetic tone (experiments 1 and 4), but significantly reversed the ptosis produced by blocking the vagosympathetic trunk (experiment 3). The dilute concentration of phenylephrine used in this study was shown in a pilot study to have a minimal effect when used in the normal eye of horses with Horner's syndrome while still significantly reversing the ptosis in the affected eye. The effect of the palpebral nerve block was determined by assessing the menace response, in which the efferent pathway is mediated by CN VII. It is clinically significant that the ptosis score of animals with a markedly reduced or absent menace response secondary to blocking the facial nerve was modest or absent in four of the six subjects, reinforcing the observation that suspected paresis of eyelid muscles innervated by that nerve is best assessed by palpation rather than by assessing the degree of ptosis. A thorough ocular examination should also ensure that any change in the angle of the eyelashes is not due to pain or enophthalmos.

Phenylephrine is a direct-acting adrenergic agonist, but unlike epinephrine and norepinephrine it is α_1 receptor selective (Hoffman and Jefkowitz, 1996), and therefore causes contraction of the smooth muscles of the eyelid. Dapiprazole on the other hand specifically blocks α_1 adrenergic receptors (Valeri *et al*, 1986) and is used to reverse the mydriasis induced by sympathomimetic and parasympatholytic compounds. Ptosis is a recognised side effect in the use of dapiprazole in a small proportion of human patients (Wilcox *et al*, 1995), however the marked effect on the equine eyelid was greater than expected and may be associated with the additional presence of the *arrectores ciliorum*.

The effect of sedation on phenylephrine response was evaluated in order to assess its potential impact on the diagnostic use of alpha agonist eyedrops in clinical cases with ptosis. Both phenothiazines and the α_2 agonist drugs are known to have muscle relaxation properties and this was borne out by the increased response of the eyelids to phenylephrine in sedated animals. Phenothiazines interfere with the central actions of catecholamines and peripherally block cholinergic, adrenergic and ganglionic activity (Muir, 1991) by blocking postsynaptic α_1 receptors (Hoffman and Jefkowitz, 1996) leading to muscle relaxation. The α_2 agonists such as xylazine and romifidine bind central and peripheral presynaptic inhibitory α_2 receptors, hyperpolarising neurons (Hedrick *et al*, 1995) and inhibiting norepinephrine release. Both α_1 and α_2 receptors are stimulated peripherally by the α_2 agonist sedatives, however detomidine has 100 times more α_1 affinity than xylazine (Muir, 1991). It is possible that in a larger study detomidine would be found to have a lesser impact than xylazine on palpebral paresis. The degree of ptosis in this study did not correlate with degree of sedation, as exemplified by the acepromazine treated animals which did not appear to be sedated but responded conspicuously to phenylephrine administration, suggesting less effective sedative but greater muscle relaxation properties of α_1 receptors.

In small animal and humans patients, the presence of Horner's syndrome is confirmed clinically by evaluating pupillary responses to topical direct-acting noradrenergic agonists (O'Brien, 1991). Horses have been shown to have a variable or absent pupillary response both to sympathetic denervation and to topical administration of sympathetic agonists (Smith and Mayhew, 1977; Hacker *et al*, 1987; Simoens *et al*, 1990; Gelatt *et al*, 1995) and this study was consequently not designed to quantify the response of pupils. Subjective assessments of pupillary responses throughout the current experiments however are in line with these previous studies; namely pupillary responses to adrenergic agonist or antagonist eyedrops were minimal if at all present, and only inconsistent miosis was noted on inducing Horner's syndrome in these animals.

This study shows that ptosis is not an inevitable consequence of acute palpebral nerve pathology and that a decrease in sympathetic tone causes paresis of the smooth

This study shows that ptosis is not an inevitable consequence of acute palpebral nerve pathology and that a decrease in sympathetic tone causes paresis of the smooth musculature of the palpebra and eyelashes. Alpha agonist eyedrops are a useful additional diagnostic test in detecting ptosis induced by sympathetic denervation in unsedated horses.

3. CRANIAL NERVE III MORPHOLOGY

a) Introduction

Ptosis is evident in AGS cases of short duration and remains a prominent clinical sign even in chronic cases after many weeks. If the cause of the ptosis is pathology to the neurons of CN III, it is reasonable to expect alterations in the peripheral nerve either because of primary axonal or myelin damage, or secondary to neuronal cell death.

Chromatolysis is classically equated with a reaction due to axonal disruption (Summers *et al*, 1995), which raises the question of whether the chromatolysis noted in EGS brainstem LMNs is due to a primary axon pathology. Hodson, (1984b) ultrastructurally examined myelinated preganglionic sympathetic fibres from EGS cases and noted myelin lamellar stripping and vesiculation, in addition to accumulations of dense bodies in Schwann cells. The changes were suggested to be similar to retrograde lesions following axonal pathology. Gilmour (1975) described changes resembling neuraxonal dystrophy of postganglionic sympathetic axons in the stellate ganglion of EGS cases, and after observing accumulations of nor-adrenaline in the abnormal structures in the axon tracts deduced that there is a primary axonal lesion in grass sickness (Gilmour, 1976). The complete loss of Golgi apparatus recognised by Griffiths (1993) in postganglionic sympathetic neurons however has never been observed in somatic or visceral neurons after experimental axotomy.

Longitudinal 1μm semi-thin sections of carefully preserved oculomotor nerve from EGS and control cases were examined to try to answer the question of whether CN III pathology is a cause of ptosis in EGS, and to further assess whether somatic LMN cell death is occurring in this disease.

b) Materials and Methods

Oculomotor nerves were obtained from two control horses and three cases of EGS (Table 4-9). The nerve was collected by removing the cap of the calvarium as soon as possible after euthanasia, usually within 20 minutes, and severing CN III close to the orbital fissure when removing the brain. The proximal portion of the nerve was cut close to its origin at the base of the mesencephalon. Nerves were suspended in 3% glutaraldehyde in cacodylate buffer by fixing one end from the brim of a plastic universal with a hypodermic needle, and weighting the nerve with a 0.4g weight attached to a size 12 de-barbed fish hook. After four days the weights were removed and the nerves kept immersed in the glutaraldehyde until further processing.

Tissues were processed by the Applied Neurobiology Group, University of Glasgow. Samples were trimmed to remove the ends and washed in 0.1 M sodium cacodylate buffer before post-fixing in 1% osmium teroxide in 1% potassium dichromate and 0.85% sodium chloride at pH 7.2 for 45 minutes. Samples were washed further in sodium cacodylate, stained with toluidine blue for one minute, and dehydrated in increasing concentrations of alcohol. Finally the tissues were infiltrated in increasing concentrations epoxy resin (Araldite, Agar Scientific Ltd) in propylene oxide for a total of 30 hours, infiltrated in Araldite Mix and Araldite Mix with accelerator (AGAR Scientific) and embedded for 48 hours at 60°C in Araldite Mix with Accelerator. Semi-thin 1μm longitudinal and transverse sections were cut using an ultramicrotome (Reichert OMU4 ULTRACUT, Leica UK Ltd) and mounted routinely on one glass slide.

Sections were examined under a microscope for evidence of histopathology (Dyck 1993).

Table 4-9: Subjects included in ptosis study

Case #	Signalment	Diagnosis	Duration (days)
99/279	4 yo WB gelding	CGS	5
99/330	4 yo Highland pony mare	CGS	20
99/299	4 yo WB gelding	SGS	5
98/820	3 yo WB mare	CVM	
98/738	9 yo Clydesdale gelding	Orthopaedic	

c) Results

The sections examined were judged to be well preserved. Axon material was variably maintained depending on the section. Occasional axons were not truly longitudinal but the majority could be adequately assessed while in the plane of section. Schmidt-Lanterman incisures could be differentiated and indentations of myelin by fibroblast and Schwann cell nuclei was evident. Nodes were difficult to distinguish and occasional artefactual vacuoles were present. There was no evidence in any sections of decreased myelin density, macrophage activity, myelin ovoids or disruption of myelin above that which could be explained by artefact.

d) Discussion

Disorders of the peripheral nerve can be classified in terms of the components of the neuron that is primarily affected, thus a primary loss of the neurons contributing axons to the peripheral nerve is categorised as a neuronopathy, while pathology to the axon may be due to primary axonopathy or secondary to a demyelinating neuropathy (Thomas *et al*, 1993).

Alterations due to axonal degeneration are the basis of the classical wallerian degeneration resulting in characteristic lesions at histopathology, while changes due to myelin pathology have been well defined in teased fibre studies (Dyck, 1993). A detailed assessment of myelin pathology in EGS fibres would be optimised by the use of a teased fibre protocol, however the process which is most likely to cause peripheral nerve alteration in EGS is axonal degeneration following neuronal death. The mechanism by which somas and their axons degenerate in neuronal degeneration

is incompletely known (Dyck, 1993) but axonal pathology in sural nerve biopsies from ALS patients has been described to include acute axonal degeneration, with initial widespread "wrinkling" of myelin and uneven myelin thickness eventually resulting in myelin degeneration into linear rows of myelin ovoids and balls (Dyck *et al*, 1975; Sobue *et al*, 1981; Bradley *et al*, 1983). It seems likely that these are secondary effects resulting from axonal degeneration caused by deterioration and loss of somatic LMNs in the spinal cord (Perrie *et al*, 1993).

The absence of pathological changes evident in semi-thin sections in this study strongly suggests that neuronal cell death or axon degeneration is not taking place in the oculomotor nucleus of EGS cases. However, axonal histopathological changes due to neuronopathy may be not be noted on histopathology as only a very low proportion of abnormal fibres may be present (Dyck *et al*, 1975). The detection of subtle pathological changes may require the use of morphometric techniques, performed in the next section of work.

4. CRANIAL NERVE III MORPHOMETRY

a) Introduction

This technique allows the appraisal of the diameter of axons, the myelin thickness and fibre densities, as well as calculated derivatives, particularly the diameter distribution of fibres and the ratio of the axon diameter (d) to the total myelinated fibre diameter (D), the 'g' ratio. Loss of a specific functional or physiological group of fibres can be expected to alter some or all of these parameters. While there is a striking difference in fibre composition among nerves and a great variety in diameter histograms among species, the median diameter and diameter peaks of a specific nerve at a given level are remarkably consistent. Morphometric values are sufficiently consistent to evaluate the class of neuron, the level within the neuron, and the components (axon or myelin) affected (Dyck, 1993).The technique is well established in some species, particularly man, in which a large amount of comparative data on normal subjects are available.

Routine histopathologic evaluation in horses is restricted to specific diseases such as equine motor neuron disease (Jackson *et al*, 1996) and idiopathic laryngeal hemiplegia (Duncan *et al*, 1978). The recurrent laryngeal nerve of horses with idiopathic laryngeal hemiplegia has been comprehensively assessed (Duncan *et al*, 1978; Duncan and Brook, 1985; Cahill and Goulden, 1986a), however evaluation of the nerve in normal control horses (Lopez Plana *et al*, 1993) is hampered by the high incidence of subclinical disease (Cook, 1988). The only comprehensive examination of an equine peripheral nerve was undertaken by Wheeler (1987), who closely examined a somatic sensory nerve, the lateral palmar nerve.

Comparison of oculomotor nerve data to other nerves necessitates the classification of this cranial nerve as a peripheral nerve, an issue that is not without controversy in the literature, as many authors classify cranial nerves separate from other nerves particularly when applied to clinical disease (Anthoney, 1993). The evaluation of general somatic efferent cranial nerves is usually limited to the facial nerve (Fahrenkamp and Friede, 1987; Thurner *et al*, 1993) and very few studies in any

species have quantitatively investigated the oculomotor nerve. One study evaluating the the components of the nerve roots of the oculomotor nerves of ALS and recessive bulbospinal muscular atrophy cases was unable to uncover any evidence of pathology (Sobue *et al*, 1981). Bronson (1978), used the oculomotor nerves of rats to evaluate the use of light and electron microscopic morphometric techniques and different statistical tests performed on nerve data. The axon distribution was found to be bimodal, and formal methods of dividing the distribution into component normally distributed parts were used to derive the mean diameters of the upper and lower peaks. Large and small classes of axons in the oculomotor nerve were also noted in a further study designed to examine morphometric axon-myelin relationships, however diameter summary data were not provided (Fraher, 1989).

This study was designed to evaluate axon and fibre diameters and distribution curves in the oculomotor nerve of control and EGS cases in order to detect evidence of pathology in the principal nerve responsible for innervation of the *levator palpebrae superioris*.

b) Materials and Methods

(i) Case material

Oculomotor nerves (CN III) were collected from 26 EGS cases and control horses in 1997 and 1998. Samples from 13 cases were processed to semi-thin (1 μm) sections and the eight highest quality sections were chosen for morphometry (Table 4-10).

Table 4-10: Experimental animals

Case #	Signalment	Diagnosis	Duration (days)
98/179	4 yo Connemara female	CGS	35
98/264	4 yo TBx gelding	CGS	16
98/303	6 yo Cob gelding	SGS	3
98/401	9 yo Arab x female	SGS	5
98/263	1 yo TB female	AGS	1
98/365	6 yo Irish Draft female	Colic	
98/677	5 yo TBx gelding	Oesophageal stricture	
98/699	3 yo Pony x male	Strangulated scrotal hernia	

(iii) Tissue materials and Methods

The nerve was collected by removing the cap of the calvarium as soon as possible after euthanasia, usually within 20 minutes, and CN III was severed close to the orbital fissure when removing the brain. The proximal portion of the nerve was cut close to its origin at the base of the mesencephalon and stretched onto a piece of cardboard and immersed in 4% formalin for between one and three days.

Tissues were processed by the Electron Microscope Unit, R(D)SVS. Samples were washed in 0.1M sodium cacodylate buffer three times for 20 minutes each and postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 45 minutes. This standard method resulted in inadequate fixation of the centre of the nerve, and it was found that initial fixation of the nerve in 1% osmium tetroxide, with further immersion of 2mm thick pieces of nerve for 40 minutes, improved axonal differentiation in some of the samples by allowing the fixative to penetrate through the myelin sheathes. Samples were then washed three times in distilled water, stained with toluidine blue for 30 seconds and dehydrated through increasing concentrations of ethanol. After placing in a 50:50 Araldite/dodecanyl succinic anhydrate (DDSP) mixture (AGAR Scientific) three times in dry apparatus, they were left overnight at 60°C. The following day they were infiltrated in Araldite Mix and Araldite Mix with

accelerator (AGAR Scientific) for 60 minutes each before finally embedding for 48 hours at 60°C in Araldite Mix with accelerator. Semi-thin (1 μm) transverse sections were cut using an ultramicrotome (Reichert OMU4 ULTRACUT, Leica UK ltd) and up to six sections were mounted on one glass slide. Addition of a coverslip using Permount was found to decrease diffraction under light microscopy.

(iv) Quantitative studies

Semi-thin sections of nerve were magnified to x 400 and captured from a microscope (Nikon Optiphot-2) through an attached analogical RGB videocamera (TK 1070E, JVC). Images were digitised with a frame grabber (Snapper-24, Data Cell) connected to a computer. The diameters of fibres and axons were measured with the help of a computerised image analysis system (Image-Pro Plus, Media Cybernetics), calibrated using a slide micrometer. The resolution was set to 1024 x 768 pixels and the corresponding yield was 3.56 μm/pixel. A variation on systematic random sampling of squares (Mayhew and Sharma, 1984) was used to obtain a systematic random pattern of fields covering each montage. The first field was chosen at one extreme of the section and a repeating pattern was chosen to cover the section (see Figure 4-4). The pattern chosen allowed at least 600 fibres per sample to be measured for the first five samples and greater than 300 for three further samples after analysis of the initial data. The repeatability was verified by counting one section twice on different days using this system but with a different starting co-ordinate, to result in two independent and non overlapping observations.

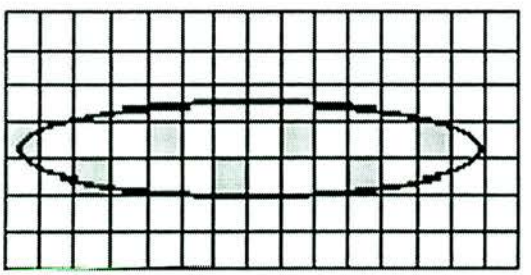


Figure 4-4: Systematic random sampling of axons from semi-thin sections of nerve

Myelinated fibres and their axons in each field were outlined individually on a digital graphics tablet (Ultraslate, Coopertina). Tangentially sectioned or artificially changed fibres were excluded from the analysis. After separating automatically linked outlines, the diameter of a circle of the equivalent area was derived for each measurement. Axon and fibre outline data were paired by sorting the grid coordinates of the centre of the perimeters in Excel (Microsoft Inc). Statistical software (C-stat, Oxford Scientific) was employed to determine the relationship between axon and fibre diameters in samples from grass sickness and control cases. Fibres were not measured if they appeared crenated or deformed. Descriptive statistics were calculated and diameter distributions were determined and plotted graphically. The g - ratios, indicating the thickness of the myelin sheath relative to the diameter of the axon, were derived from axonal and myelinated fibre diameter data as follows:

$$\text{g ratio} = \frac{\text{axonal diameter (d)}}{\text{fibre diameter (D)}}$$

c) Results

The fixation of material appeared not be optimal, however it was judged to be adequate for purposes of the present study (Figure 4-5). A median of 531 fibres were counted per sample (range 310 to 706). Median fibre and axon diameters and g - ratios of the oculomotor nerve of three control and five EGS cases are shown in Table 4-10. The g - ratio data were normally distributed and mean and standard deviations are presented (Figure 4-6). Scatter plots with a simple regression line relating fibre diameters to axon diameters for total EGS and control data are shown in Figure 4-7. Fibre and axon diameter distribution histograms for individual sample and summed control and EGS data are given in Figure 4-8 to 4-11. The fibre diameter distribution graphs were bimodal in shape with the peak of the lower mode visually assessed at 4 μm and the upper mode at 13 μm for both the summed control and the EGS data. The upper mode was much more difficult to identify in the axon distribution curves and was unimodal in some cases, particularly when the combined

control data are considered. The g - ratios from control and EGS cases however appeared to represent a single population (Figure 4-12).

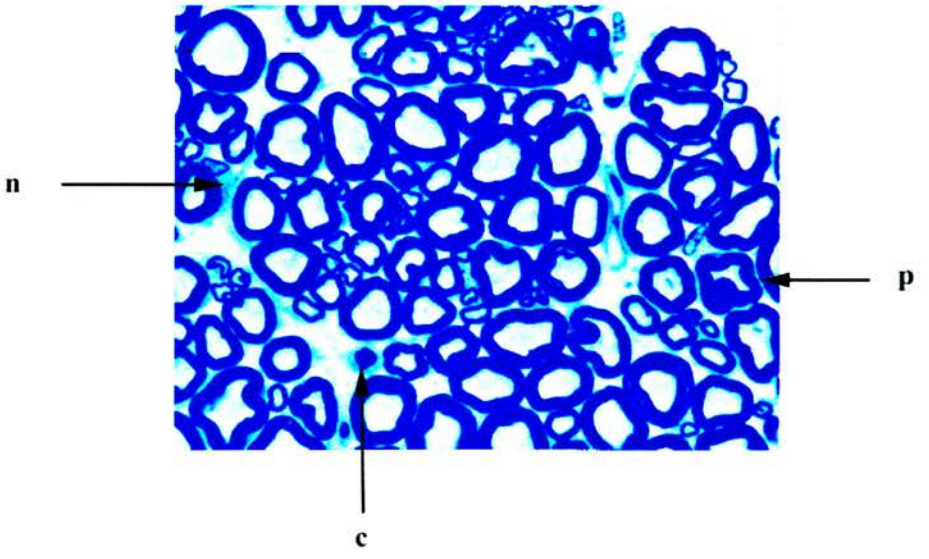


Figure 4-5: CN III, semi-thin transverse section, SGS.
The field contains numerous myelinated fibres of a range of diameters, outlined by densely stained myelin sheaths. A Schwann cell nucleus (n), a paranodal region (p) and an endoneurial capillary (c) are labelled.

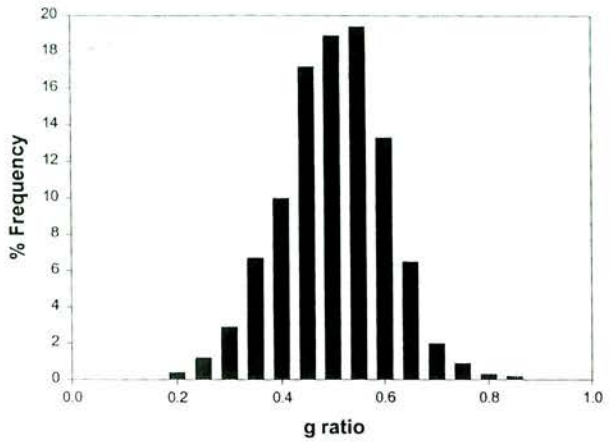
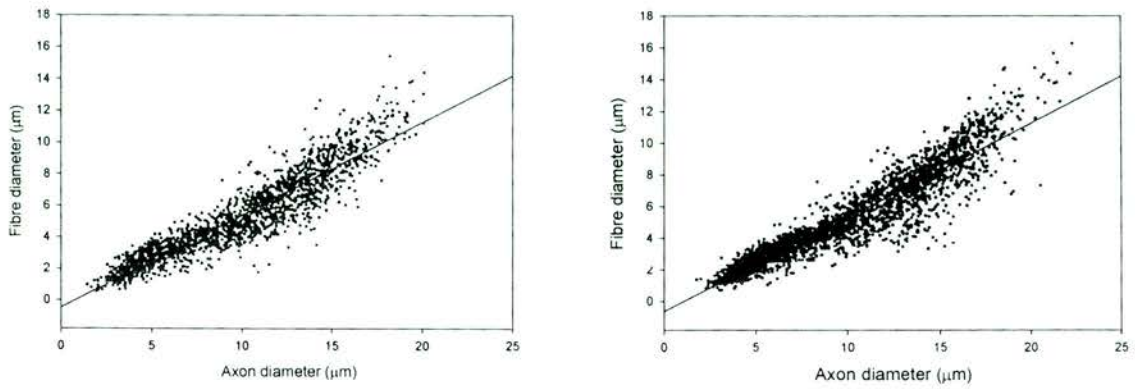


Figure 4-6: g - ratio diameter distributions (%) (all data).

Table 4-11: Median values (minimum, maximum) for fibre diameter, axon diameter and the ratio of axon to fibre diameter, mean value of g - ratio (± standard deviation)

Horse	Diagnosis	Count	Median fibre diameter (μm)	Median axon diameter (μm)	g - ratio (SD)
98 677	Control	684	10.2 (1.5 - 20.1)	5.3 (0.6 – 15.4)	0.56 (0.10)
98 699	Control	706	10.2 (1.7 – 19.1)	4.8 (0.6 –13.5)	0.50 (0.10)
98 365	Control	320	10.4 (2.1 – 22.2)	4.7 (0.9 – 12.4)	0.53 (0.11)
Total control		**1710**	**10.3**	**5.0**	**0.53 (0.11)**
98 263	AGS	628	8.9 (2.4 – 20.6)	4.4 (0.7 – 14.1)	0.50 (0.09)
98 264	CGS	628	10.7 (2.7 - 22.3)	5.3 (1.2 - 16.3)	0.54 (0.08)
98 179	CGS	661	9.0 (2.4 – 20.8)	4.4 (0.7 – 16.3)	0.51 (0.09)
98 401	SGS	310	9.9 (1.8 – 19.0)	4.2 (1.0 –14.7)	0.47 (0.10)
98 303	SGS	311	9.8 (1.9 – 19.2)	4.3 (0.8 – 14.6)	0.48 (0.11)
Total EGS		**2538**	**9.5**	**4.5**	**0.52 (0.10)**



Control EGS

Figure 4-7: Scatter plot with simple regression line relating fibre diameter to axon diameters. Control (N = 1710) and EGS (N = 2538) cases.

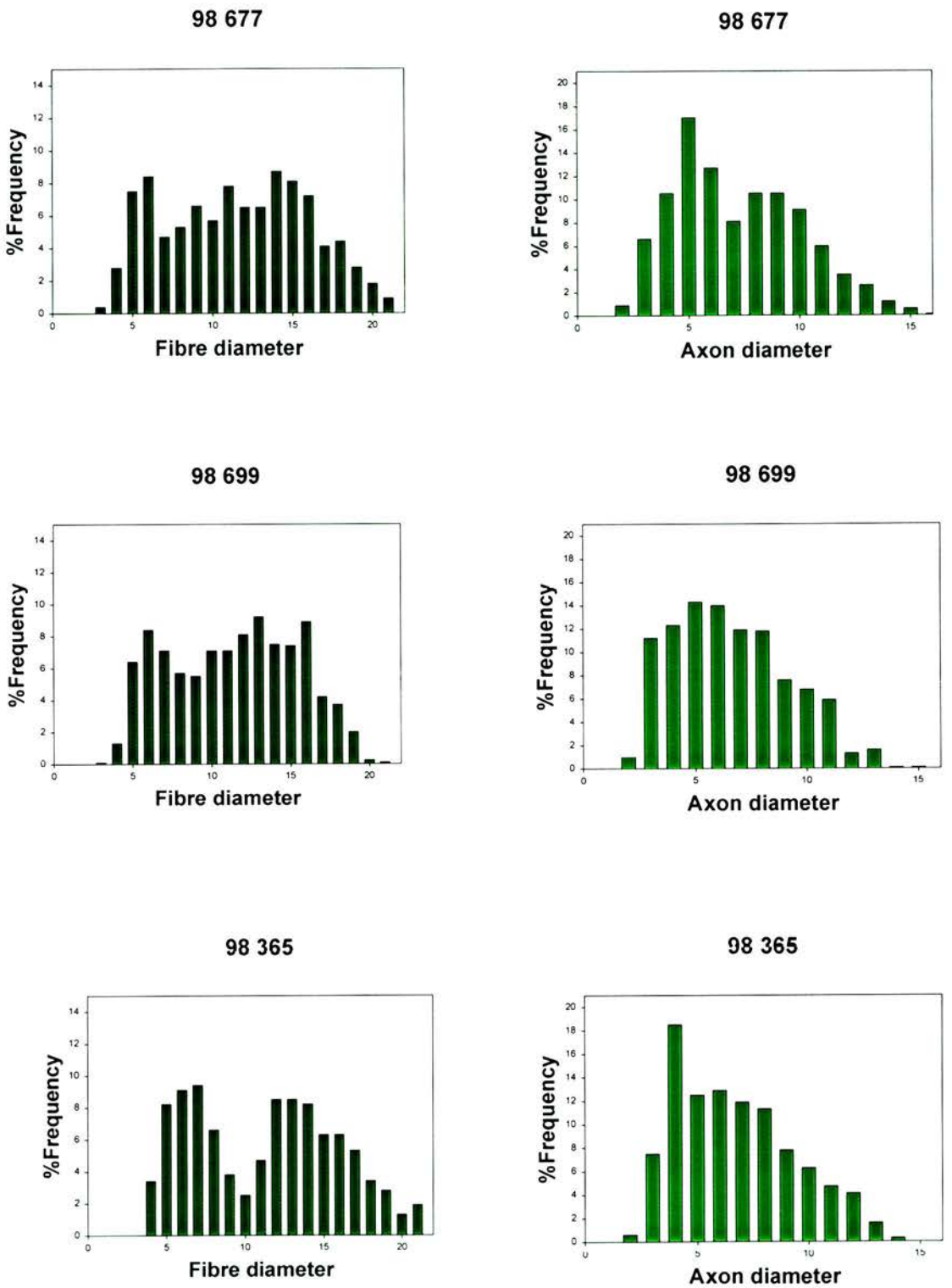


Figure 4-8: CN III fibre and axon diameter distributions (%). Control animals.

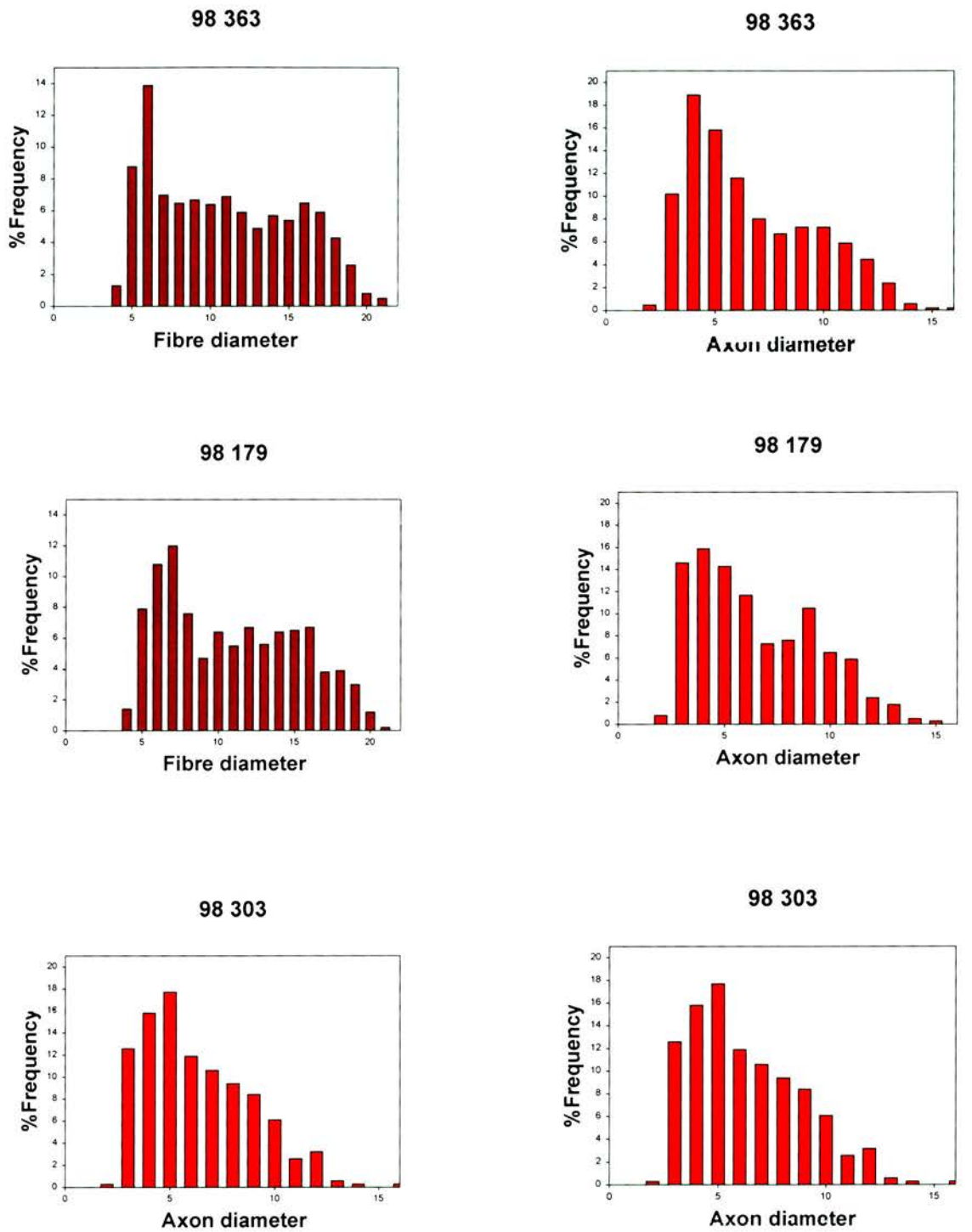
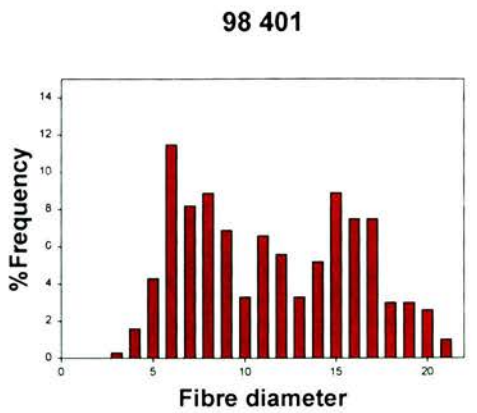
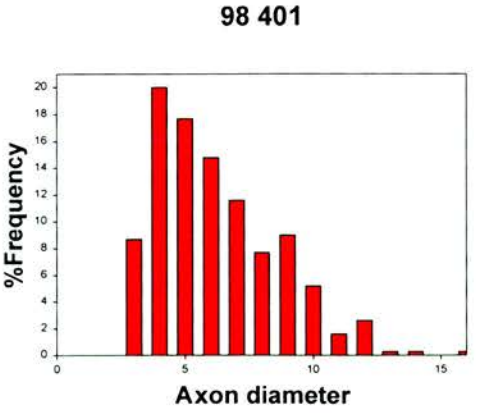
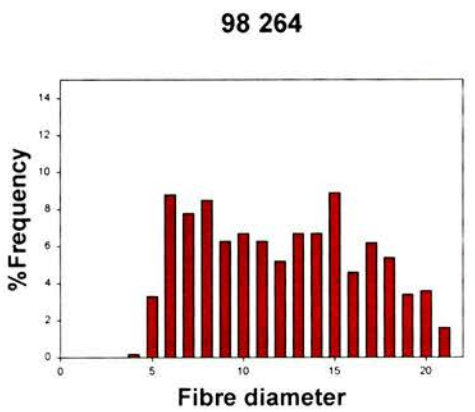
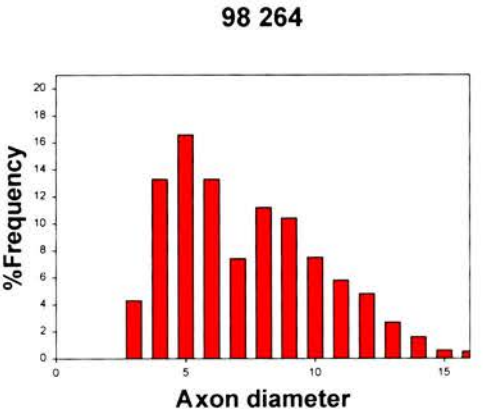


Figure 4-9: CN III fibre and axon diameter distributions (%). EGS cases









(Figure 4-9 continued)

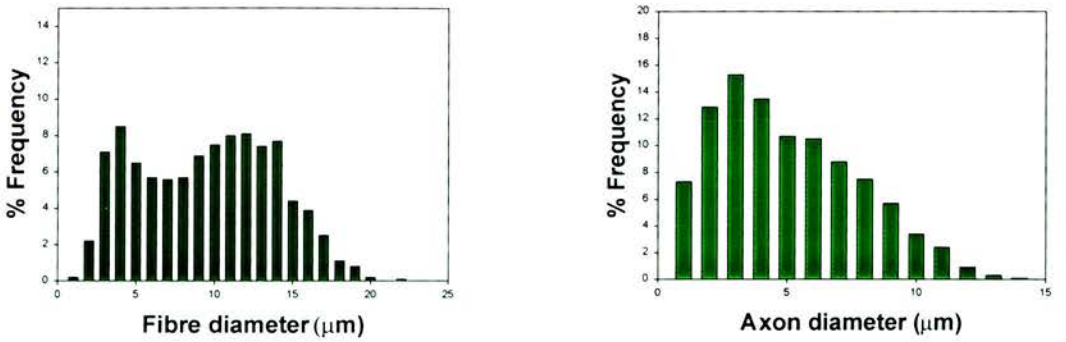


Figure 4-10: CN III fibre (left) and axon (right) diameter distributions (%). All data, control animals.

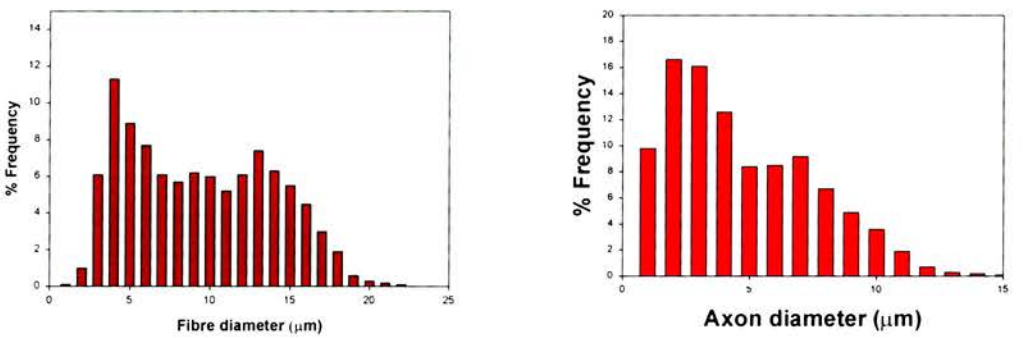


Figure 4-11: CN III fibre (left) and axon (right) diameter distributions (%). All data, EGS cases

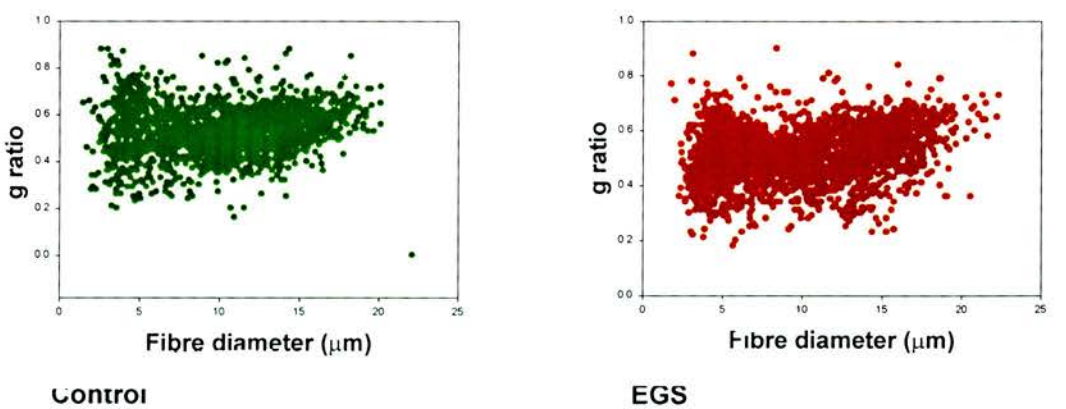


Figure 4-12 g - ratio by fibre diameter from control (left) and EGS cases (right)

d) Discussion

A number of fixatives have been recommended to preserve nerves for morphometry including differing concentrations of glutaraldehyde (Duncan *et al*, 1978; Cahill and Goulden, 1986a; Vita *et al*, 1992), glutaraldehyde and paraformaldehyde (Fraher, 1989; Wheeler, 1991) and formalin (Braund *et al*, 1982b). The latter was chosen with advice from that author but did not result in optimal fixation of the myelin sheathes and precluded analysis of unmyelinated fibre diameters. Axons have been shown to have a greater diameter in paranodal regions, thus paranodal fibres, as well as severely crenated fibres were excluded from the analysis (Braund *et al*, 1982b). The state of fixation prevented the categorical identification, and exclusion, of Schmidt-Lanterman incisures, however these have been noted to account for less than 1% of all fibres (Mayhew and Sharma, 1984) and have a minimal effect on external fibre diameters (Jacobs and Love, 1985). The number of fibres counted per sample in this study is in line with the sample size recommended in the literature (Braund *et al*, 1982b; Wheeler, 1991) and the systematic random sampling of squares method for counting fibres has been shown to be highly efficient and to give sampling trial results which are less than 1% different to absolute counts (Mayhew and Sharma, 1984). Density of axons was not assessed due to the considerable variability in both density and number of myelinated fibres found at a given level of a specific nerve (Dyck, 1993) and with different processing methods (Wheeler, 1987).

Fibre diameter data primarily appeared to have a bimodal distribution, which formally precludes the use of parametric statistical techniques. Dividing bimodal data into two normally distributed components is feasible in some cases (Bronson *et al*, 1978) but did not result in appropriate distributions for this data. The most commonly biopsied human nerve, the sural nerve, has a bimodal distribution (Jacobs and Love, 1985; Dyck, 1993), as have equine lateral palmar nerves (Wheeler and Plummer, 1989) and rat oculomotor nerves (Bronson *et al*, 1978). Biimodal distributions are a characteristic of nerves such as feline trochlear and abducens nerves (Hildebrand *et al*, 1988), human facial nerves (Thurner *et al*, 1993), canine

nerves (Hildebrand *et al*, 1988), human facial nerves (Thurner *et al*, 1993), canine medial articular nerve (O'Connor *et al*, 1982) and equine (Duncan *et al*, 1991b; Lopez Plana *et al*, 1993) and canine (Braund *et al*, 1982b) distal laryngeal nerves. Proximal portions of the recurrent laryngeal nerve and the vagus on the other hand have a unimodal distribution (Braund *et al*, 1988b), and canine peroneal and ulnar nerve frequency distributions were found to be unimodal at birth and bimodal at 3-6 months old (Braund *et a*l, 1982b), emphasising the importance of controlling for the specific biopsy site and the age of the subject.

Neurodegenerative diseases characteristically result in a loss of distinct fibre populations (Sobue *et al*, 1981; Hanyu *et al*, 1982; Bradley *et al*, 1983; Cahill and Goulden, 1986a; Atsumi and Miyaatake, 1987; Rosales *et al*, 1988; Braund *et al*, 1994), shifting the median and modal peak diameter values. The very similar median and equivalent modal and peak diameter values of EGS and control fibres in this study indicate that they are unlikely to be based on different fibre size populations. The median diameters of fibres in this study is similar to that found by Duncan (1991b) in an analysis of distal equine recurrent laryngeal nerve, and the modal peak diameters correspond to those determined in the equine lateral palmar nerves (Wheeler and Plummer, 1989). Median fibre diameters however are significantly larger than the mean diameters obtained from human and rat samples of the oculomotor nerve (Bardosi *et al*, 1990).

g - ratios can be a sensitive means to indicate the presence of peripheral neuropathy; high g - ratios being associated with regeneration (Beuche and Friede, 1985) while abnormally low g - ratios are a feature axonal atrophy (Jacobs and Love, 1985), as exemplified by uraemic neuropathy (Dyck, 1993). The most reliable way to determine g - ratios is by counting myelin lamellae using ultrastructural techniques, but it is satisfactory to calculate them by measuring axon and fibre diameters (Vita *et al*, 1992). The g - ratios of the same nerve can vary greatly between species (Bardosi *et al*, 1990), and within one species can differ significantly even between fibre classes of nerves that are closely similar in respect of morphological class, central origin, peripheral distribution and function (Fraher, 1989). The g - ratio values

determined in this study are within the limits of normal values found in adult human sural nerve biopsies (Jacobs and Love, 1985). The g - ratios of human and rat oculomotor nerve fibres (Fahrenkamp and Friede, 1987; Bardosi *et al*, 1990) can be grouped into two main populations, differing in their relative myelin sheath thicknesses. The general relationship of g - ratios and fibre size is unclear however, with different studies finding that g - ratios increase, decrease or remain unchanged with increases in axon diameters (Thomas *et al*, 1997). Grouping of g - ratios was not apparent in this study however, suggesting that the ratio of myelin to fibre diameters was constant in both control and EGS samples. This provides further indirect evidence that axonal atrophy (and thus neuronal death) or hypertrophy is not taking place in CN III axons in EGS cases and that pathology of CN III is not the cause of ptosis in EGS cases.

5. MORPHOMETRY OF THE EQUINE LEVATOR ANGULI OCULI MEDIALIS MUSCLE

a) Introduction and Literature Review

The *levator anguli oculi medialis* (LAOM) muscle (Figure 4-1) is a thin, small muscle which arises over the root of the zygomatic process and spreads out in the upper eyelid, blending in with the *orbicularis oculi.* It is innervated by the auriculopalpebral branch of cranial nerve VII and assists raising the upper eyelid of equids (Getty, 1975). Paresis of the LAOM following pathology to CN VII would be shown to be a cause of ptosis in EGS cases if changes compatible with denervation were demonstrated in that muscle.

The muscle is subdivided into fascicles by a well defined collagen layer called the perimysium. Individual muscle fibres are multinucleate syncytia surrounded by a cell membrane, the sarcolemma, and are separated from one another by a network of fine collagen fibres, the endomysium (Dubowitz, 1985; Loughlin, 1993).

Individual fibres are divided into three fibre types with distinct metabolic and functional features. Type I fibres are characterised by a relatively strong aerobic capacity, are associated with sustained activity and are well represented in postural muscles. Type II fibres are involved in rapid, powerful movements and are subdivided into Type IIA fibres, which have been found to be more glycolytic with strong aerobic and moderate-to-strong anaerobic capacity, and Type IIB fibres which are characterised by a relatively low aerobic and a relatively high anaerobic capacity.

The histochemical differentiation of fibres into two main types, as well as at least two subtypes of Type II fibres, is based on adenosine triphosphatase (ATPase) reactivity with ATPase substrate under acidic and alkaline preincubation to inactivate different ATPase systems. The ATPase enzyme catalyses the reaction ATP + H2O → ADP + orthophosphates + energy. The tissue is incubated in a solution containing ATP and calcium at pH 9.4, splitting off the terminal phosphate from ATP if the ATPase is active. The phosphate immediately combines with calcium to form

insoluble calcium phosphate which is deposited at the site of the enzyme activity. The addition of cobalt chloride solution forms cobalt phosphate which is turned into black cobaltous sulphide by a reaction with ammonium sulphide. The ATPase present in various muscle fibres is highly dependent upon the influence of pH and the reaction can be used to demonstrate distinct fibre types by preincubating the tissue at different pH values. The ATPase enzyme has been found to be more reliable than other enzyme systems for fibre typing in diverse physiological and pathological states (Dubowitz, 1985; Braund, 1991; Loughlin, 1993)

The appearance of muscle pathology secondary to denervation, and the use of qualitative and quantitative diagnostic methods have been well reviewed (Dubowitz, 1985; Loughlin, 1993). Although the changes are not specific for denervation (Johnson *et al*, 1973b), classical histopathological signs include the presence of angular fibres, greater than 3% of fibres containing internal nuclei, small or large group atrophy and somal changes including pyknotic nuclear clumps and pale staining hyaline (necrotic) fibres.

More subtle changes may only be disclosed by the complementary use of quantitative methods. The combination of denervation and compensatory increase in work load by remaining fibres commonly results in muscle fibre diameters alterations, frequently shown only by an increase in range of sizes without an associated change in mean diameters. The fibre size variability coefficient, defined as the fibre size data standard deviation x 1000 divided by the mean fibre diameter, is considered to be abnormal if it exceeds 250 (Dubowitz, 1985; Loughlin, 1993) to 300 (Braund, 1991). This does not appear to have been substantiated in equids.

A further use of quantitative methods is in the analysis of fibre type arrangements and fibre type grouping. A reinnervated muscle fibre will become the fibre type of the motor unit which adopts it (Dubowitz, 1970), leading to a loss of random arrangement of fibre types. Assuming that Types I and II fibres are arranged randomly throughout a muscle, the number of fibres of one type which may be found lying in contact with each other in normal muscle is related to the proportions of the

two main fibre types. A muscle is considered to be enclosed if, in transverse section, it is completely surrounded by fibres of its own histochemical type. This usually only occurs if at least five fibres of the same type are together (Jennekens *et al*, 1971). The proportion of enclosed fibres will increase as the proportion of that fibre type in muscle rises, but remains at a relatively low prevalence until greater than 70% of fibres in a muscle are of one type (Loughlin, 1993). The distribution of fibre types is random in the great majority of muscles (Johnson *et al*, 1973a) and a number of statistical models have been devised to calculate the expected number of enclosed fibres (Johnson *et al*, 1973b; Loughlin, 1993). A representative computer simulation designed to model fibre type grouping was proposed (Lexell *et al*, 1983).

Increasingly it has become clear that that morphological and morphometric changes must be interpreted in the light of a sound appreciation of the range of normal variation. The pathological features of denervation and reinnervation can, to a lesser extent, also be observed in control subjects (Iwasaki *et al*, 1991) and considerable variation in mean diameters, coefficients of variation, distribution and proportions of fibre types have been found associated with variables such as the specific muscle examined (Jennekens *et al*, 1971), depth of biopsy within a specified muscle (Mahon *et al*, 1984; van den Hoven *et al*, 1985b), gender (Moore *et al*, 1971; Loughlin, 1993), age (Moore *et al*, 1971; Essen *et al*, 1980; Lexell and Downham, 1991; Loughlin, 1993) and duration of fixation (Moore *et al*, 1971).

The technique for histochemical determination of equine muscle fibre types and the metabolic profiles of healthy equine *gluteus medius* muscle has been described (Hodgson *et al*, 1983; van den Hoven *et al*, 1985a). Fibre characteristics and enzyme activities were determined for the *gluteus, semitendinosus, vastus lateralis* and *triceps brachii* muscles of a large number of Standardbred trotters of different ages, focusing on the proportion of fibre types and the histochemical staining characteristics. In all the muscles examined, Type II fibres predominated (69 – 87%) and, with the exception of the *vastus lateralis*, was age related (Essen *et al*, 1980). Further work concluded that large differences existed in the same zone of individual

muscles and it was concluded that results obtained by biopsies of muscles must be interpreted with caution (van den Hoven *et al*, 1985b).

Very little work, however, has determined values for fibre diameter, variability and fibre type grouping data. The majority of work on equine muscle has focused on a few specific diseases such as EMND (Valentine *et al*, 1998) and particularly idiopathic laryngeal hemiplegia (ILH) (Duncan *et al*, 1977). The interpretation of histopathological changes of the intrinsic musculature of the equine larynx in horses affected with ILH (Cahill and Goulden, 1986b; Duncan *et al*, 1991a), clinically normal horses (Gunn, 1972), foetal horses (Gunn, 1973; Harrison *et al*, 1992) and particularly distal limb muscles of ILH and control cases (Gunn, 1972; Cahill and Goulden, 1986b; Kannegieter, 1989) has been impeded by a lack of sufficient data on the normal morphometry of equine muscle fibres.

Neither the LAOM muscle nor its analogue in the bovine, the frontalis muscle, have been examined in detail. The purpose of this study was to assess the normal histology, morphometry and fibre size variability of the LAOM and to determine fibre type grouping in terms of random and non-random arrangements to disclose any subtle evidence of neurogenic pathology in EGS cases.

b) Materials and Methods

The LAOM was collected from four adult horses with no clinical evidence of facial nerve or LAOM pathology, and six horses with a confirmed diagnosis of EGS (Table 4-12). A transverse section of the muscle was dissected as soon as possible after euthanasia, adhered to a cork carrier and frozen immediately in isopentane in dry ice to a temperature of -96°C. After freezing the blocks were stored at -80°C until analysis, at which time 8 μm thick transverse serial sections were cut onto Biobond coated glass slides (British Biocell International, Cardiff, UK) and allowed to air dry.

Table 4-12: Subjects included in LAOM morphometry study

ID	Diagnosis	Days affected	Signalment
98 339	AGS	5	15 yo Connemara mare
98 372	AGS	2	16 yo TB mare
98 401	SGS	6	9 yo Arab x mare
98 443	AGS	3	4 yo TB gelding
98 461	AGS	3	2 yo TB gelding
99 279	CGS	12	3 yo Welsh gelding
98/365	Colic		6 yo Irish Draft mare
98/738	Orthopaedic		7 yo Clydesdale gelding
98/820	CVM		1 yo WB mare
99/522	Colic		18 yo Arab x mare

(i) Histology

One cryostat section for each sample was stained with hematoxylin and eosin and examined for histopathological evidence of denervation including the presence of angular fibres, small group atrophy and internal nuclei. Nuclei were defined as internal if they were further than two nuclear diameters from the cell wall. The number of muscle fibres per fascicle was quantified in tissue sections from control animals.

(ii) Enzyme histochemistry

Individual fibre types were demonstrated using calcium activated myofibrillar ATPase. Pilot studies in our laboratory have found better segregation of Type IIA and IIB fibres at pH 4.3 rather than pH 4.6 recommended in the literature (Dubowitz, 1985). Sections were fixed in cacodylate buffered formalin for two minutes and rinsed in distilled water before being incubated in acetate buffer at pH 4.3 for 15 minutes. After rinsing in distilled water, sections were immersed in freshly prepared 37°C ATPase incubation solution containing ATP disodium salt and calcium chloride in tris acetate buffer at pH 9.4. Sections were incubated for between 20 and 30 minutes and then rinsed in distilled water, immersed in 2% cobalt chloride for 2 minutes, rinsed again and developed in fresh 1% ammonium sulphide before being mounted routinely. This technique results in Type I fibres being stained dark brown,

while Type IIA fibres are pale and Type IIB fibres have intermediate staining properties (See Figure 4-13).

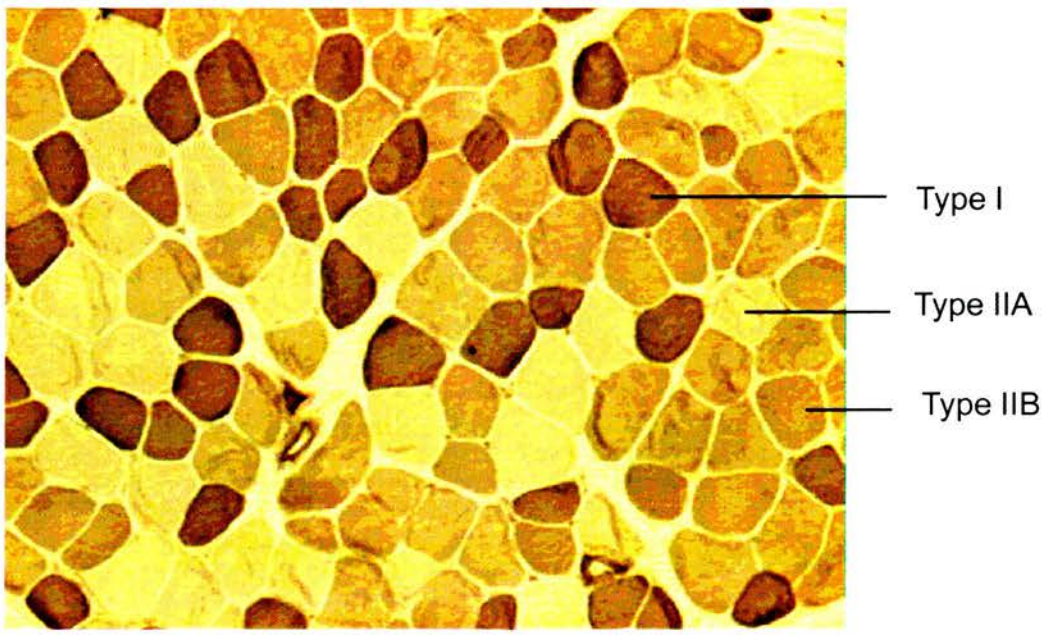


Figure 4-13: Transverse section LAOM ATPase histochemistry. pH 4.3, CGS

(iii) Quantitative techniques

Sections were magnified to 100x, captured from a microscope, digitised, and the images downloaded onto a personal computer. Morphometry was performed with the help of a calibrated computerised image analysis system (Image-Pro Plus, Media Cybernectics).

Four or five fields containing only transverse sections of fibres were chosen to cover the whole section and including a minimum of 350 and a maximum of 600 fibres. Fibres were manually outlined using a digital graphics tablet (Ultraslate, CalComp

Technology, Inc.) and divided into Type I, Type IIA and Type IIB fibres using histogram based segmentation after contrast enhancement. All fibres in a field which were not touching the edge were included and the area, number and diameter of fibres was recorded. Descriptive statistics were calculated and diameter distributions were determined and plotted graphically. The fibre size variability coefficient was calculated as

$$\frac{\text{fibre size data standard deviation x 1000}}{\text{mean fibre diameter}}$$

The data were visually assessed and the distribution judged to be approaching normality (Figure 4-14). Students t-test and analysis of variance techniques were applied when appropriate. Non parametric techniques were used on proportion data and 95% confidence intervals calculated from the Binomial distribution (Rohlf and Sokal, 1981).

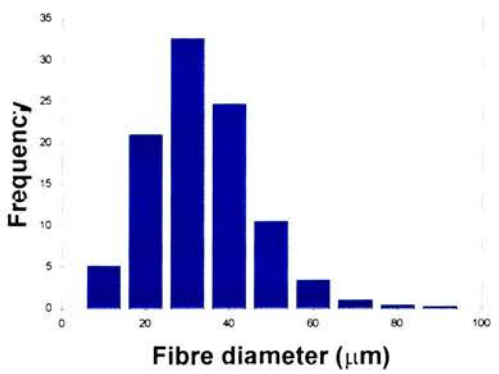


Figure 4-14: Fibre diameter frequency histogram, all data

(iv) Fibre type grouping

All fields included in the quantitative fibre type analysis were re-examined and the number of enclosed fibres noted. An enclosed fibre was defined as one that was enclosed by fibres of the same type along its major surfaces, excluding the corners.

To determine fibre type grouping in terms of random and non-random arrangements, a model by Lexell (1983) was used to calculate the expected number of enclosed fibres. Briefly, the model assumes a binomial distribution of the number of enclosed fibres and the expected number of enclosed fibres for a particular fibre type is equal to MP^7, where M equals the number of internal points in a field, assuming that each fibres has six neighbours, and P is the proportion of fibres that are of a particular fibre type. M is the largest integer less than $n - 2 \times \sqrt{\pi n}$ where n is the number of fibres of a specified type. The proportion of the most commonly occurring fibre type was determined for control or EGS samples and P substituted accordingly.

c) RESULTS

(i) Histology

No signs of denervation were noted in tissue sections from control or EGS cases. Specifically there was no evidence of groups of angular atrophied fibres, multiple internal nuclei, proliferated sarcolemmal nuclei or inflammatory cells. A single internal nucleus was found in an occasional fibre. Fibres with multiple internal nuclei, or split fibres, were very rare and angular fibres were not noted. Muscle spindles were not observed. Thirty eight randomly chosen fascicles in control tissues yielded a mean of 163 (+/- 72) fibres per fascicle.

(ii) Proportion of fibre types

The total number and proportions of Type I, IIA and IIB fibres are given in Table 4-13, Table 4-14 and Figure 4-15. ANOVA on ranks and pairwise multiple comparison procedures indicated that the proportion of Type IIA fibres was

significantly smaller than the proportion of Type IIB fibres in both EGS ($p<0.001$) and control samples ($p = 0.04$). There was no significant difference between type I fibres and Type IIA or IIB fibres in EGS or control cases. Within the same fibre type there was no significant difference between EGS and control animals (Rank sum t-test, all $p > 0.2$).

Table 4-13: EGS LAOM fibre numbers (proportion) by fibre type

ID	Total #	Type I (prop)	Type IIA (prop)	Type IIB (prop)
98/443	607	198 (0.33)	114 (0.19)	295 (0.49)
98/339	462	138 (0.43)	62 (0.13)	202 (0.44)
98/372	361	91 (0.25)	37 (0.10)	233 (0.65)
98/401	345	96 (0.23)	84 (0.24)	165 (0.48)
98/461	526	158 (0.30)	93 (0.18)	275 (0.52)
98/279	431	106 (0.25)	55 (0.13)	270 (0.63)
All	2732	787 (0.29)	445 (0.16)	1440 (0.53)

Table 4-14: Control LAOM fibre numbers (proportion) by fibre type

ID	Total #	Type I (prop)	Type IIA (prop)	Type IIB (prop)
99/522	603	157 (0.26)	127 (0.20)	319 (0.53)
98/820	540	164 (0.30)	54 (0.10)	322 (0.60)
98/365	407	83 (0.20)	64 (0.16)	260 (0.64)
98/738	469	166 (0.35)	44 (0.09)	259 (0.55)
Total	1986	570 (0.29)	289 (0.15)	1127 (0.57)

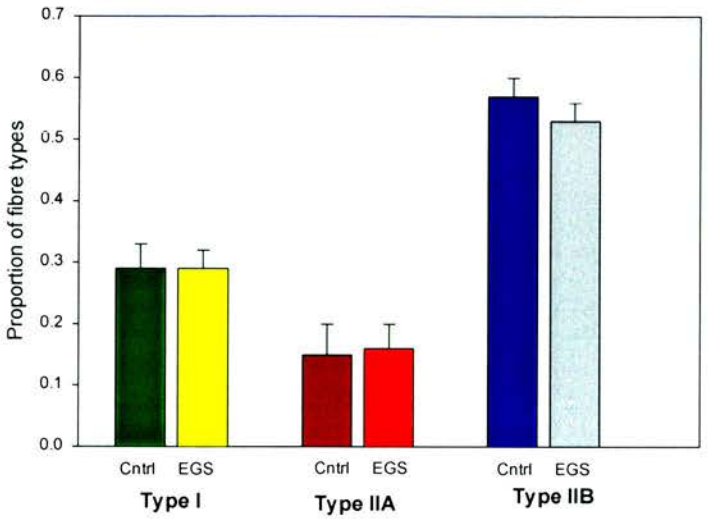


Figure 4-15: Proportions of fibre types in control and EGS cases. Median plus 95% CI

(iii) Fibre diameters

The summed diameter distributions by fibre type for EGS and control horses are given in Table 4-15. Summary statistics are shown in Figure 4-16. The mean diameters of Type I ($p = 0.004$), Type IIA ($p = <0.001$) and Type IIB ($p = 0.003$) fibres were statistically significantly different between EGS and control horses. Fibre size variability coefficient data are given in Table 4-16.

Table 4-15: Fibre diameter (μm) summary statistics

	Type I Mean (SD)	Type IIA Mean (SD)	Type IIB Mean (SD)
EGS LAOM	31.0 (8.49)	44.1(12.24)	41.21(13.89)
Control LAOM	29.7 (8.53)	41.1 (10.05)	39.6 (13.03)

Table 4-16: Fibre size variability coefficient

	Type I	Type IIA	Type IIB
Control	287	246	328
EGS	274	277	337

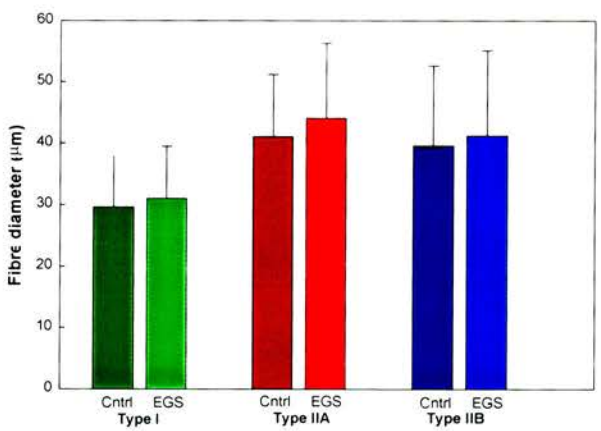


Figure 4-16: Diameter distributions by fibre type.
Mean plus standard deviation

(iv) Fibre type grouping

Type IIB fibres represented the highest proportion of fibres present and were the only type in which enclosed fibres were noted. The proportion (P) of Type IIB fibres determined in EGS and control cases was substituted accordingly. The number of enclosed fibres noted, and the number of enclosed fibres expected using the statistical model are shown in Table 4-17. The number of expected and calculated enclosed fibres were not significantly different when control ($p = 0.343$) or EGS ($p = 0.15$) data were compared. There was no significant difference (Rank Sum ANOVA $p = 0.272$) between any of the samples when data for all fields in all samples was compared.

Table 4-17: Number of expected and actual enclosed fibres

ID		n	M	Number expected	Number actual
98/522	Cntrl	319	256	5	2
98/820	Cntrl	322	258	5	10
98/365	Cntrl	260	203	4	7
98/738	Cntrl	259	202	4	9
98/443	EGS	295	234	3	2
98/339	EGS	202	152	2	2
98/372	EGS	233	179	2	12
98/401	EGS	165	119	1	2
98/461	EGS	275	216	3	3
99/279	EGS	270	212	2	10

n = number of fibres counted. M = model number internal points in a field.

(v) Internal nuclei

The number of fibres on the section with internal or multiple nuclei was assessed. The fascicles present on that section were counted and the total number of fibres present was calculated using the mean number of sections per fascicle determined (Table 4-18). A median of 0.1% of all fibres contained at least one internal nucleus. Multiple internal nuclei were very rarely noted. There was no significant difference ($p = 0.9$) between the proportion of internal nuclei in control and EGS fibres.

Table 4-18 Proportion of fibres with internal nuclei, estimating the number of fibres per section from the estimated mean number of fibres/fascicle

	Nucleated fibres	No. of fascicles	Estimated no. of fibres	Proportion of fibres with internal nuclei
98/365 control	45	6	10,000	0.005
98/522 control	14	8	13,000	0.001
98/738 control	30	11	18,000	0.002
98/820 control	13	7	11,000	0.001
98/372 EGS	19	11	18,000	0.001
98/401 EGS	60	10	16,000	0.004
98/339 EGS	71	10	16,000	0.004
98/461 EGS	20	10	16,000	0.001
98/502 EGS	17	11	18,000	0.001

d) Discussion

There was no histopathological evidence of neurogenic atrophy in any of the EGS tissues examined. The duration of clinical disease was arguably very short for a number of the acute cases examined, however the severity of enteric and ganglionic neuronal pathology in that group of cases implies that the disease process has been ongoing for significantly longer time than suggested by the presence of clinical signs. Morphologic signs compatible with the neurogenic atrophy however would have been expected in sections from the CGS case if denervation of the muscle had occurred.

The median figure of 0.1% of fibres with internal nuclei is small compared to quoted data (Braund *et al*, 1988a; Braund, 1991) (Dubowitz, 1985; Loughlin, 1993) and is notable in that a ten fold increase in incidence in internal nuclei may be a sign of significant pathology in this muscle, but would lie well within the normal range in the muscles more commonly examined. The difference may lie in the fact that muscles usually chosen for biopsy, and hence histopathological examination, are active postural or locomotion muscles which may be subject to greater stresses than the LAOM.

The predominance of Type II fibres in the LAOM is not unexpected in view of the high proportion of that fibre type in locomotory muscles (Valentine *et al*, 1998). Indeed it has been suggested that the act of blinking may be one of the best examples of phasic activity and the *orbicularis oculi,* the main eyelid muscle innervated by the facial nerve, contains a high proportion of Type II fibres (Johnson *et al*, 1973a).

The diameters and frequency distributions of Type I and Type II fibres vary among different muscles and are also influenced by factors such as age, gender, nutritional status and degree of physical activity (Loughlin, 1993), further emphasising the importance of specific data being available for each muscle examined. Essen (1980) as a general rule suggested that Type IIA fibres are usually 1.5 to 2 times larger than Type I fibres, which is in agreement with this data. The total number of fibres examined in this study compares favourably with the 150-200 fibres per case which is accepted to give a reproducible and consistent value for mean diameters and standard deviations (Jennekens *et al*, 1971; Dubowitz, 1985; Loughlin, 1993). Control mean fibre diameter data were statistically significantly different to data from EGS cases, however in light of the small actual difference (Figure 4-16) is unlikely to be biologically significant.

In many instances an increase in range of sizes is a more sensitive indicator of myopathies than a change in mean diameters (Loughlin, 1993), nevertheless artefactual variation may account for the minor differences in fibre size variability coefficient noted in these data. Mahon (1984) found considerable variations in the

proportions of fibre types and mean fibre size between individuals in duplicate needle biopsies from the *quadriceps femoris* muscle from healthy, age matched human males. Within individuals, comparisons of samples taken at a reference site with samples obtained from deeper, more proximal or contralateral sites also often showed significant differences in fibre size and suggests that caution is necessary when interpreting apparent changes in such values. Data for fibre diameters from both control and EGS cases were slightly greater than the suggested maximum in normal humans (Dubowitz, 1985; Loughlin, 1993), however the observation that the variability coefficient for the same muscle in dogs alters with the size of subject (Braund, 1991) implies that further work on horses is required before these figures are more stringently interpreted.

The presence of fibre type grouping is contingent on reinnervation having taken place, which is dependent on the capacity of the unaffected motorneurons to reinnervate adjacent denervated muscle fibres. This varies between specific neurodegenerative diseases (Telerman-Toppet and Coers, 1978), and fibre type grouping does not appear to be a feature of a prominent equine neurodegenerative disease, EMND (B. Valentine, Oregon State University, personal communication). The presence of uniform fibre type grouping however, may not be justified as a definite index of reinnervation, for example if replacement fibres are innervated by collateral sprouting or if innervation is initiated by axons of both types (Johnson *et al*, 1973b). Nevertheless, the lack of fibre type grouping in the LAOM from EGS cases provides evidence that denervation followed by reinnervation has not taken place.

The lack of evidence of LAOM denervation does not categorically rule out that paresis of the LAOM is occurring, however purely functional pathology to the lower motor neuron soma or axon is extremely rare. A number of toxins such as the ionophore antibiotics, numerous chemotherapeutic agents, heavy metals and pesticides (Innes and Saunders, 1962) are associated with peripheral neuropathy, but would be expected to produce generalised clinical signs and structural neuropathology, with subsequent neurogenic atrophy of innervated muscles. Toxins

affecting presynaptic neuromuscular transmission produce paresis, however these are not generally associated with a somal reaction and would be expected to cause generalised paresis. Similarly, acquired metabolic diseases are not expected to target nuclei with such extreme specificity.

In conclusion the results of this study suggest strongly that CN VII neuronal cell death is not occurring and is not the cause of ptosis in EGS.

6. ELECTROMYOGRAPHIC EVALUATION OF BLINK REFLEX LATENCIES

a) Introduction

Neuronal death in neurodegenerative disorders may be preceded by periods of neuronal dysfunction (Waggie *et al*, 1999), and electrodiagnostics can be used to provide detailed information about the function of the neurologic system by recording spontaneous and induced cell electrical activities (Andrews and Fenner, 1987).

The blink reflex is a contraction of the *orbicularis oculi* muscle due to reflexly activated motorneurons of the facial nerve, and is used in human medicine to study the integrity of trigeminal and facial nerve function for the evaluation of peripheral neuropathies and pontine and rostral medullary syndromes (Kimura, 1992). The reflex pathway includes the supraorbital nerve branch of cranial nerve V, which synapses with the nucleus of cranial nerve VII in the rostral medulla. Efferent impulses reach the *orbicularis oculi* muscle through the facial nerve, giving an early ipsilateral oligosynaptic response designated R1. Later ipsilateral and bilateral R2 responses are thought to represent afferent impulses entering the pons through the trigeminal nerve, descending in the spinal trigeminal tract along the dorsolateral brain stem to at least the second cervical spinal cord segment before ascending to make connections with the ipsilateral and contralateral facial nuclei (Eekhof *et al*, 1996). The R2 response can be abolished with severe cranial spinal cord lesions (Nukes *et al*, 1995). A third R3 response can be observed, and is significantly related to pain sensation in man (Rossi and Vignocchi, 1993). The R1 response is more stable than the ipsilateral or contralateral R2 responses, and is suitable for studies of the function of the afferent and efferent pathways (Kimura, 1992) .

Blink reflex latencies were studied in EGS cases to try to assess integrity of neurons in CN VII supplying the auriculopalpebral branch of the facial nerve, the same nerve that innervates the *levator anguli oculi medialis*.

b) Materials and Methods

Blink reflex latencies were determined in 17 EGS cases and 19 unaffected horses. Control animals were either experimental ponies or animals in the hospital having electrodiagnostic tests done for other reasons.

Compound motor action potentials (CMAPs) were recorded after stimulating the supraorbital nerve by tapping the zygomatic process with an external triggering hammer. This triggered an electrodiagnostic unit (Neurostar MS92B, Medelec Ltd, Old Woking, UK) and the resulting evoked compound muscle action potentials were recorded from the ipsilateral *orbicularis oculi* muscle by a needle electrode inserted subcutaneously in the lower eyelid. The reference electrode was sited over the ipsilateral facial crest and the ground electrode was inserted into the musculature of the cranial neck. These sites were chosen as those which consistently give a smooth, repeatable waveform. The electrodes were connected to the signal averaging equipment via a pre-amplifier (PA92B, Medelec Ltd, Old Woking, UK) that was attached to the subject's head collar. Ipsilateral recordings were made on averaged successive stimulations, the number of tracings included in the average differing between subjects. A sweep of 100 ms was recorded with the amplitude set at 100μV per division, low frequency filter set at 2 Hz and high frequency filter at 10 kHz. The electromyographically recorded responses consist of polyphasic early R1 response in the *orbicularis oculi* muscle and, variably, a late R2 response.

Each subject was measured to obtain an estimate of the length of the reflex pathway. The measurements were taken with a flexible steel tape measure from the stimulus site to the base of the ear, and from there to the recording electrode inserted into the *orbicularis oculi*.

Latency was defined as the time interval (msec) between the start of recording by the triggering hammer to the initial deviation from baseline in the positive direction, and separately to the apex of the positive peak of R1. In a few control cases data for the initial deviation from the baseline was not measured. In cases which had an inverted trace with a major negative peak, negative reflections from baseline and negative

peak latencies were measured. If the peak reflex latencies were different for separate traces, the visual median between peaks was determined and noted. Data were excluded if the initial deviation from baseline could not be recorded due to artefact secondary to environmental electrical activity or muscle movement. Latency velocities were calculated by dividing the measured distances by the latency.

c) Results

Stable and repeatable R1 responses were obtained with little apparent increase in latency due to habituation (Figure 4-17). R2 responses were inconsistently observed and R3 responses were never noted. Estimated lengths of the reflex pathway, the latency of the initial deviation from the baseline and to the peak of R1, and the calculated reflex latency velocities of control and EGS subjects are given in Table 4-19 and Table 4-20. Summary data are shown in Table 4-21 and Table 4-22.

All data passed formal normality tests. There were no significant differences between control subject and EGS case mean latencies of the deviations from the baseline ($p = 0.87$), peak latencies ($p = 0.47$) or reflex latency velocities ($p = 0.32$). There was no correlation between the estimated length of the pathway and the peak latency ($p = 0.4$), or the latency of the deviation from the baseline ($p = 0.6$).

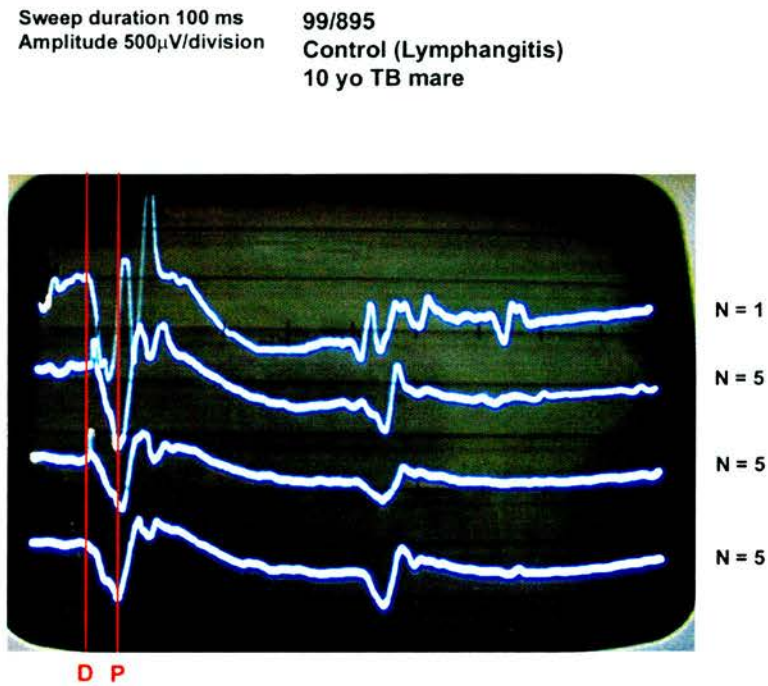


FIgure 4-17: Recording from the ipsilateral *orbicularis oculi* muscle showing R1 response and R2 responses
D = deviation from the baseline latency, P = peak latency, N = number of averaged sweeps

Table 4-19: Blink reflex data control animals

		Latencies ($m.sec^{-1}$)		Reflex latency velocity (ms)	
Case #	Length (m)	Deviation from baseline	Peak	Deviation from baseline	Peak
1	0.29	6.0	10.0	48.3	29.0
2	0.34	7.0	16.0	48.6	21.3
3	0.37	10.4	15.0	35.6	24.7
4	0.28	6.5	11.5	43.1	24.3
5	0.23	8.5	15.0	27.1	15.3
6	0.32	11.0	17.0	29.1	18.8
7	0.28	9.0	12.5	31.1	22.4
8	0.3		12.0		25.0
9	0.25		9.0		27.9
10	0.25		12.4		20.2
11	0.27		11.6		23.3
12	0.26		11.6		22.4
13	0.26		13.2		19.7
14	0.37	6.4	9.6	57.8	38.5
15	0.36	6.0	13.2	60.0	27.3
16	0.33	4.8	19.6	68.8	16.8
17	0.30	4.4	20.0	68.2	15.0
18	0.22	10.0	14.0	22.0	15.7

Table 4-20: Blink reflex data EGS cases

		Latencies (ms)		Reflex latency velocity ($m.sec^{-1}$)	
Case #	Length (m)	Deviation from baseline	Peak	Deviation from baseline	Peak
97/363	0.23	6.5	10.5	35.4	21.9
97/528	0.23	8.0	16.0	28.8	14.4
97/987	0.37	7.0	13.0	52.9	28.5
98/244	0.28	7.0	10.4	40.0	26.9
98/408	0.28	3.2	12.0	87.5	23.3
98/431	0.23	7.0	10.0	32.9	23.0
98/448	0.27	13.0	14.5	20.8	18.6
98/501	0.25	9.0	14.0	27.8	17.9
98/502	0.31	11.5	14.5	27.0	21.4
98/540	0.24	10.0	13.0	24.0	18.5
98/877	0.27	5.2	10.0	51.9	27.0
97/834	0.27	5.0	11.0	54.0	24.5
98/335	0.23	7.2	17.0	31.9	13.5

Table 4-21: Control horse blink reflex results summary

	Mean	SD
L (m)	0.29	0.05
Deviation from baseline (ms)	7.5	2.22
Peak (ms)	13.5	3.13
Peak reflex latency velocity ($m.sec^{-1}$)	45.0	16.3
Deviation from baseline reflex latency velocity ($m.sec^{-1}$)	22.64	5.84

N=18

Table 4-22: EGS blink reflex results summary

	Mean	SD
L (m)	0.27	0.04
Deviation from baseline (ms)	7.7	2.69
Peak (ms)	12.8	2.34
Peak reflex latency velocity ($m.sec^{-1}$)	40.0	18.1
Deviation from baseline reflex latency velocity ($m.sec^{-1}$)	21.5	4.72

N=13

d) Discussion

Mean peak R1 latencies determined in this study were 5 ms shorter than others reported in horses (Añor *et al*, 1996; Añor *et al*, 1999). A difference in latencies can be expected however since the latter studies evoked the response using electrical stimulation sited over the supraorbital nerve, and recorded the CMAP with surface electrodes. The more direct stimulation of the afferent limb of the reflex in that study however, may have been expected to result in shorter latencies.

Blink reflex latencies are assessed in man without attention to the size of the subject (Kimura, 1992). Since the size of the head varied greatly between ponies and horses in this study, latency velocities were calculated in order to standardise the technique for different sizes of animals. However there was no correlation between latencies and estimated lengths of the pathway, which substantiates the sole use of latencies in equids as applied in other studies (Añor *et al*, 1996; 1999).

A CMAP is the result of many muscle fibres being concurrently active. Large axonal fibres, responsible for the latency of the deviation from the baseline (Añor *et al*, 1999), are capable of discharging at a faster rate than small fibres as shown by the faster initial deviation from the baseline reflex latency velocities. Latencies may be normal during early phases of axonal or myelin damage (Sims, 1994), however the reduction in the number of innervated motor units should result in a decrease in observed amplitude and increased duration of the CMAP (Aminoff, 1999), and ideally the duration and amplitude of the CMAPs in this study would be determined. In line with conclusions in the literature however (Aminoff, 1999), the amplitude of the R1 response were noted to vary greatly between subjects in this study and thus were not quantified.

The lack of a statistical difference between EGS and control animal latencies of the deviation from baseline or peak of the CMAP is strong evidence of an intact pathway in EGS cases, and indicates that a CN VII functional deficit is not involved in ptosis in EGS cases.

7. PHENYLEPHRINE EYEDROPS AS A DIAGNOSTIC TEST IN EQUINE GRASS SICKNESS

a) Introduction

In light of the severe pathology seen in the cranial cervical ganglia of EGS cases, it was hypothesised that the ptosis seen in these animals is predominantly due to sympathetic denervation. Neuronal damage in the cranial cervical ganglion of EGS cases is extensive (Pogson *et al*, 1992), but morphometric techniques to assess axonal degeneration of postganglionic sympathetic fibres, as well as being technically difficult (Gibbels, 1989; Wheeler and Plummer, 1989) would not determine if there is a functional effect on eyelid tone. Ptosis in horses due to sympathetic denervation was shown to be reversible with alpha agonist eyedrops (chapter 4 section 2) and the administration of 10% phenylephrine eyedrops to horses with EGS was found to result in a dramatic reduction of ptosis, lasting for several hours. 10% phenylephrine however, the concentration used in the diagnosis of Horner's syndrome in small animals and humans, also resulted in marked effects on the eyelids of control horses. A pilot study determined the optimal concentration of phenylephrine and the time required to elicit a significant decrease in ptosis EGS cases, while giving the least increase in eyelash angle of normal control horses.

The current quantitative study was performed to establish whether ptosis in EGS cases is a manifestation of Horner's syndrome and to determine if this technique has potential as a simple, non-invasive, inexpensive pre-mortem diagnostic test for grass sickness.

b) Materials and Methods

Twenty-three EGS cases and 12 horses with other diagnoses were included in this study (Table 4-23). Control animals were chosen as horses that might have had EGS but subsequently were found to have other diseases. Phenylephrine (Phenylephrine 10% w/v, Martindale), diluted to 0.5% with normal saline, and 0.5 ml of this stock was applied to the conjunctiva of one eye and the subjective size of the palpebral

fissure and angle of the eyelashes to the cornea was evaluated 30 minutes later by comparing the treated and control eyes. Frontal photographs were taken using a 1.4 Mega pixel digital camera (Olympus D-600L) and analysed using a computerised image analysis system (Image-Pro Plus, Media Cybernetics). The difference in angles of the eyelashes to a line drawn between the medial canthi of the eyes were measured. Data were analysed with the help of statistical software (SigmaStat, SPSS Science).

c) Results

Phenylephrine administration resulted in marked reduction of ptosis in EGS cases and had a minimal effect on control animals (Figure 4-18). The signalment, clinical syndrome, days affected (EGS cases) and the difference in eyelash angles of horses included in this study are shown in Table 4-23. A negative eyelash angle difference indicates a smaller palpebral fissure size in the treated eye. Summary statistics are shown in Figure 4-19. The data were normally distributed and there was a significant difference between the eyelash angle differences of EGS cases and control horses ($p < 0.001$), but within the control animal group there was no significant difference ($p = 0.14$) between orthopaedic cases (horses 1 - 4) and 'sick' animals (5 – 12). A One way ANOVA found no significant difference between AGS, SGS or CGS groups ($p = 0.71$).

There was no significant correlation (Pearson Product Moment Correlation: $p = 0.25$) between the effect of phenylephrine on the difference in eyelash angle in EGS cases and the number of days the horse was affected on the day the test was performed (median 6 days, range 1 – 65).

Table 4-23: Animals included in the study and the difference in eyelash angle between eyes after administration of 0.5% phenylephrine into one eye.

Horse	Signalment	Diagnosis	Days affected (EGS)	Eyelash angle difference
1	16 yo TB mare	Orthopaedic		-18
2	9 yo Welsh gelding	Orthopaedic		21
3	7 yo TB x gelding	Orthopaedic		-8
4	11 yo gelding	Orthopaedic		3
5	6 yo TB x TR mare	Choke		21
6	10 yo Cob x mare	Colic		9
7	7 yo Cob gelding	Colic		3
8	11 yo Cob mare	Colic		10
9	7 yo WB gelding	Colic		-5
10	7 yo TB x mare	Colic		34
11	3 yo Highland mare	Botulism		25
12	3 yo WB gelding	Dysphagia		22
13	4 yo Cob gelding	AGS	1	12
14	4 yo TB x mare	AGS	1	36
15	8 yo TB x mare	AGS	2	22
16	1 yo Highland filly	AGS	2	24
17	2 yo WB stallion	AGS	2	32
18	10 TB x stallion	SGS	3	48
19	3 yo TB mare	SGS	3	57
20	9 yo Highland mare	SGS	4	11
21	4 yo ID x mare	CGS	4	21
22	4 yo WB gelding	SGS	5	32
23	9 yo Cob gelding	SGS	6	25
24	5 yo Pony gelding	CGS	6	28
25	5 yo Pony gelding	SGS	7	35
26	3 yo Cob gelding	CGS	7	66
27	1 yo TB x mare	CGS	8	50
28	4 yo Highland mare	CGS	11	32
29	7 yo Highland gelding	CGS	12	26
30	6 yo Eriskay gelding	CGS	16	33
31	3 yo TB x gelding	CGS	17	63
32	16 yo Cob gelding	CGS	30	9
33	3 yo Cob stallion	CGS	30	40
34	8 yo Highland mare	CGS	61	18
35	6 yo TB gelding	CGS	65	15

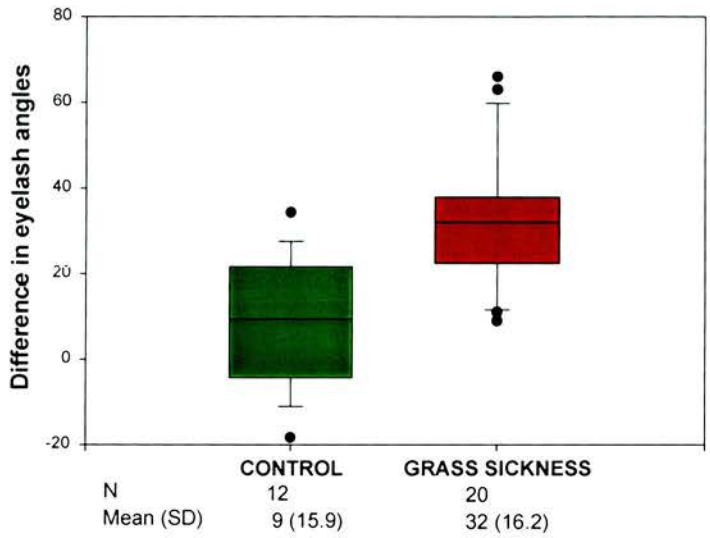


Figure 4-19: Difference in eyelash angles summary data
Key: The ends of the boxes define the 25th and 75th percentiles with a line at the median and error bars defining the 10th and 90th percentiles.

d) Discussion

The current technique of using topical ophthalmic alpha-adrenergic agonist drugs in clinical cases with ptosis represents a novel approach to the differentiation of autonomic and somatic nerve dysfunction. Smooth muscle contraction is principally effected by α_1 receptor activation, which would be effected to a far lesser degree via Müller's muscle in EGS cases due to the substantial reduction of postganglionic sympathetic neurons in the cranial cervical ganglion. Phenylephrine is a direct acting adrenergic agonist, but unlike epinephrine and norepinephrine is a selective α_1 agonist drug that activates β receptors only at much higher concentrations (Hoffman and Jefkowitz, 1996). For interpretation of this test it is critical to ensure that animals have not been sedated, even several hours previously, since sedation will lead to increased responses (Chapter 4, section 2) and this may have inadvertently occurred in some of the control animals in this study.

The lack of association between either severity or duration of EGS with response to topical phenylephrine is perhaps unexpected since continued lack of receptor stimulation might be anticipated to result in receptor hypersensitivity (Kurvers *et al*, 1996) and an increased response. Since ptosis appears not to persist in long term recovered EGS cases it may be that reinnervation by surviving neurons, or a return to function by recovering neurons, may provide sufficient α adrenergic tone in cases with longer duration of disease. Due to the small numbers of acute and subacute cases in the current experiment the result should be interpreted cautiously.

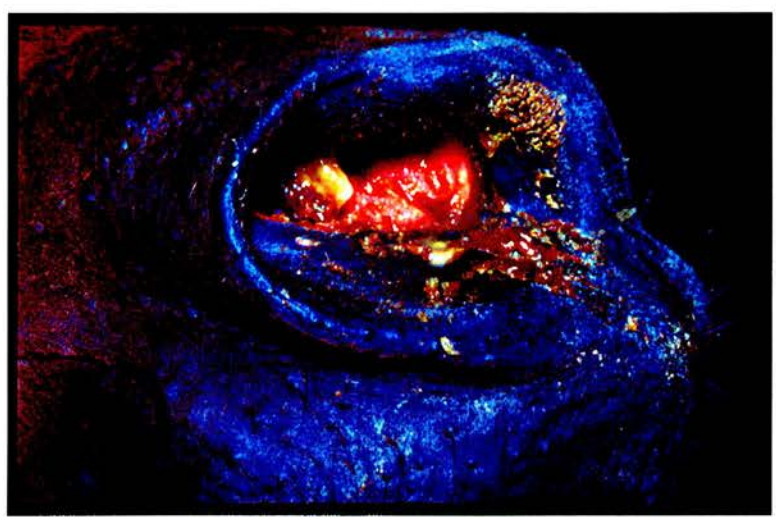
For this population there was a 25% overlap between the highest responding control animals and lowest responding EGS cases, so if an eyelash angle of 22° was taken as the cut off point between test positive and test negative animals, a specificity and sensitivity of 75% would be calculated. Of more practical benefit perhaps is the finding that only 15% of EGS cases responded at or below the mean response for control animals and we have found the technique a valuable addition to the diagnosis of suspect EGS cases. Results must of course be assessed in context of other clinical signs but we have been particularly impressed by the ability of the technique to point

out the presence of ptosis in animals which on first examination do not appear to show this very important clinical sign.

The significant difference in response to phenylephrine eyedrops between control animals and EGS cases confirms that the major or sole cause of ptosis in EGS is sympathetic neuronal dysfunction, and more importantly that this technique is a useful adjunct in the diagnosis of grass sickness.

Chapter Five

Rhinitis Sicca



1. Introduction to rhinitis sicca in EGS

Rhinitis sicca, or drying of the nasal membranes resulting in crusting of a mucoid or mucopurulent discharge on the nasal passages, is a common clinical sign in subacute and particularly chronic cases of EGS (Milne, 1991) and feline dysautonomia (Griffiths *et al*, 1982). Rhinitis sicca can appear to be distressing to the animal and severe signs are associated with a very poor prognosis (Milne *et al*, 1994). It is speculated that the rhinitis is associated with a lack of nasal secretions, but the underlying cytopathology has not been investigated.

The nasal fluid, a secretory product of the nasal mucosa, is involved in the humidification and warming of inspired air, the protection of the underlying respiratory epithelium and the removal of inhaled pollutants. The nasal mucosa is innervated by an intricate network of sympathetic and parasympathetic nerve fibres. Sympathetic axons originate in the cranial cervical ganglion, from which fibres run into the plexus of the carotid artery before joining the petrosal and vidian nerves innervating the nasal mucosa. Preganglionic parasympathetic fibres arise from the GVE nucleus of CN VII, joining the petrosal nerve to synapse with neurons in the sphenopalatine ganglion and in microganglia deep within the nasal mucosa (Klaassen *et al*, 1988). Sympathetic neurons in the cranial cervical ganglion (Obel, 1955), and parasympathetic neurons in the sphenopalatine ganglion (personal observation) show severe pathology in EGS.

Retrograde neuronal labeling of the rostro-lateral part of the nasal mucosa of rats additionally labeled neurons in the parasympathetic otic ganglion, and a large proportion of neurons in the ipsilateral trigeminal ganglion, containing neurons in the general somatic afferent (GSA) system, indicating that the latter is an important source of innervation of the nasal mucosa (Grunditz *et al*, 1994). The first order sensory neurons in the mesencephalic nucleus of V were not noted to be chromatolytic in EGS cases (chapter 2) but the same category of neuron in the trigeminal ganglion was often chromatolytic, with a very uneven distribution. The trigeminal ganglion has not been examined in detail in EGS cases, and pathology to this structure may give a further basis for the rhinitis sicca of EGS cases.

2. MORPHOMETRY OF TRIGEMINAL GANGLION AND NUCLEUS OF MESENCEPHALIC V NEURONS

a) Introduction

This study emanated from the observation that some transverse sections of trigeminal ganglion (TG) appeared to be severely affected, whereas other sections contained a minimal number of chromatolytic neurons. On examining longitudinal sections however, this appeared to depend on the number of small neurons on the field. Prominent clustering of neurons by cell sizes was occurring and sections in which the majority of neurons were smaller appeared to be more severely affected.

Mesencephalic V neurons (the only primary afferent neurons in CNS) were not noted to be chromatolytic in sections of 56 grass sickness cases examined with the exception of *two* individual achromatic neurons. This study was undertaken to determine if a relationship existed between the presence of chromatolysis and neuronal size.

b) Materials and Methods

(ii) Case material

Midbrain sections and/or trigeminal ganglia from 12 grass sickness cases and three control horses were chosen at random from those collected between 1996 to 1998. See Table 5-1.

Table 5-1: Subjects and tissues examined, Trigeminal ganglion morphometry

Case #	Signalment	Diagnosis	Duration (days)	Samples
96/425	10 yo Pony female	AGS	2	Midbrain
98/443	4 yo TB gelding	AGS	3	Midbrain
97/428	5 yo Irish Draft gelding	AGS	2	Midbrain
97/517	3 yo TB female	AGS	2	Midbrain
97/293	2 yo Highland pony female	CGS	21	Midbrain
97/313	4 yo TB female	CGS	11	Midbrain
97/348	5 yo Welsh x gelding	CGS	15	Midbrain, TG
97/453	5 yo Connemara female	AGS	2	TG
98/277	2 yo Clydesdale female	AGS	1	TG
98/461	2 yo TB gelding	AGS	3	TG
98/303	6 yo Cob gelding	SGS	3	TG
98/563	8 yo Highland pony female	SGS	3	TG
97/1116	14 yo TB gelding	Respiratory		TG
97/618	7 yo Clydesdale female	Colic		Midbrain
98/295	8 yo TB gelding	Orthopaedic		TG

Key: TG = trigeminal ganglion

(iii) Tissue materials and methods

The trigeminal ganglion was excised from the caudal portion of the canal of the trigeminal nerve on the floor of the calvarium and placed in 4% neutral buffered formaldehyde. After at least one week, longitudinal sections of trigeminal ganglion and a section of brain at the level of the caudal colliculus were blocked for processing. Sections were processed as outlined in chapter two, section 2. b.

(iv) Quantitative studies

Sections were captured from a microscope (Nikon Optiphot-2) through an attached analogical RGB videocamera (TK 1070E, JVC). Images were digitised with a frame grabber (Snapper-24, Data Cell) connected to a personal computer. The diameters of neurons was measured with the help of a computerised image analysis system (Image-Pro Plus, Media Cybernectics), calibrated using a slide micrometer. The resolution was set to 1024 x 768 pixels and the corresponding yield was 3.56 μm/pixel.

Brain stem

Groups of mesencephalic V neurons around the mesencephalic aqueduct were identified and magnified to x 40. Between two and four fields were captured to include the majority or all of the neurons on that section of brainstem. All cells that approached standard size (ie not including those which were sectioned at the very edge of the cell), whether or not they included nuclei, were outlined individually on a digital graphics tablet (Ultraslate, CalComp Technology, Inc). Descriptive statistics were calculated and diameter distributions were determined and plotted graphically (SigmaStat and SigmaPlot, SPSS Scientific).

Trigeminal ganglion

Due to the prominent clustering of cell sizes within each longitudinal section, one field was chosen for morphometry which contained mainly large neurons and another field in which neurons were mainly small neurons. Slides were magnified to x 40. Cells were outlined as above. Measurement data for each field in each case was obtained for chromatolytic and normal neurons in control and grass sickness cases .

c) Results

Total neuronal number or density could not be ascertained due to the quantitative methodology used but no qualitative decrease of neurons in EGS cases was noted and no nodules of Nageotte were evident. Combined data had a normal appearing distribution but failed the equal variance test. Individual EGS data ($p=0.11$) and control data ($p=0.06$) were not significantly different from each other. Mean diameter summary data for EGS and control trigeminal ganglion and mesencephalic V neurons are given in Table 5-2 and Table 5-3. Data is given to one decimal place to be in agreement with the accuracy of the measurement technique.

Chromatolytic TG neurons from EGS cases had the smallest mean diameters. Diameter data had a large range (control data, 24 – 72 μm) and large associated standard deviations. There was a statistically significant difference (ANOVA on ranks, $p<0.001$) in collective neuronal diameter data from control TG neurons, normal appearing EGS TG neurons, chromatolytic EGS TG neurons, control and EGS mesencephalic V neurons. Pairwise multiple comparison procedures (Dunn's method) indicated that all the groups had statistically significantly different diameter data from each other with the exception of EGS and control mesencephalic V neurons.

Table 5-2: Trigeminal ganglion neuronal diameter summary data

	Number of neurons		Mean diameter (SD)	
	Normal	**Chromatolytic**	**Normal**	**Chromatolytic**
Control cases	348	N/A	33 (11.7)	N/A
EGS	1034	296	44 (13.7)	29 (7.5)

Mean diameter measurements given in micrometers
SD = standard deviation
N/A = not applicable, no chromatolytic neurons

Table 5-3: Mesencephalic V neuron diameter summary data

	Number of neurons	Mean diameter (SD)
Control cases	38	56 (12.3)
EGS	255	48 (13.4)

Mean diameter measurements given in micrometers
SD = standard deviation

d) Discussion

Neuronal clustering by size was too extensive to allow a simple random sampling technique to be used, however counting of all neurons on two microscope fields resulted in sufficient data being collected to make the study statistically meaningful. The data of individual cases within control EGS groups were not significantly

different from each other, confirming that the morphometric methods used resulted in repeatable estimates of the sizes of neuronal cell bodies being studied. Neurons were counted whether or not they included a nucleus (underestimating cell sizes) since the presence of peripheral nuclei in chromatolytic neurons would not have permitted consistent measurements of normal and chromatolytic neurons to be taken.

Dorsal root ganglion neurons have been divided into three types of cells (A, B, C) on the basis of size, the distribution of organelles and electrophysiological characteristics (Rambourg *et al*, 1983; Wang *et al*, 1993a). Type A cells are large neurons in which Nissl bodies are evenly distributed throughout the perikaryon. Type B cells are smaller, with concentric zonation of organelles, and type C cells are the smallest, containing small, poorly demarcated Nissl bodies and a juxtanuclear Golgi apparatus. It is likely that equine dorsal root ganglion neurons can be similarly categorised by size, and this study suggests that the classification influences neuronal sensitivity to the putative EGS insult with potentially class A neurons being relatively more resistant.

In conclusion, the qualitative observation that smaller neurons were preferentially chromatolytic was quantitatively substantiated. The following section attempts to establish whether a specific marker could be used to differentiate large and small trigeminal neurons

3. Calcitonin Gene Related Peptide expression in neurons from the trigeminal ganglion and mesencephalic V nucleus

e) Introduction

Further to the investigation showing a predilection for chromatolysis by small neurons of the trigeminal ganglion of grass sickness cases, as well as the absence of chromatolysis in the large neurons comprising the mesencephalic trigeminal nucleus, a marker differentiating large and small trigeminal neurons was sought to further distinguish those neurons likely to show morphological or functional changes in EGS.

In addition to the classical neurotransmitters, an ever growing list of neuroactive peptides are recognised which modulate pre or post synaptic transmission or may use the peptide as a sole neurotransmitter. Calcitonin Gene Related Peptide (CGRP) is a 37 – amino-acid peptide expressed principally in spinal motor neurons and sensory neurons in the trigeminal and spinal dorsal root ganglia, as well as in parasympathetic ganglia and some central nuclei including the oculomotor nucleus and nuclei of CN VI, VII and XII (Silverman and Kruger, 1989; Thompson *et al*, 1995; Cooper *et al*, 1996). CGRP is present in sensory neurons and is a prominent peptide in somatic motor neurons (Thompson *et al*, 1995). From double labelling experiments it is known that nasal mucosal nerve fibres containing CGRP emanate exclusively from the trigeminal ganglion (Zhao and Tao, 1994). This is despite CGRP being present in other ganglia, such as the parasympathetic sphenopalatine ganglion (Grunditz *et al*, 1994). Distinct expression of CGRP by trigeminal neurons of different sizes, or by neurons in the mesencephalic nucleus of CN V, would be notable. In addition, there is growing evidence that the expression of CGRP is decreased in the nasal mucosa of EGS cases (Corcoran, work in progress) and it is therefore to relevant determine whether there is an association between affected (chromatolytic) and CGRP expressing neurons by comparison of morphometric data.

b) Materials and methods

(v) Subjects and tissues

Transverse or longitudinal sections of formalin fixed, paraffin embedded trigeminal ganglia from fourteen horses (two acute, four subacute and two chronic EGS cases and six control animals) were included (Table 5-4). One section from each ganglion had been assessed histologically to record the degree of chromatolysis.

Sections of formalin fixed, paraffin embedded mesencephalon containing a number of mesencephalic V nucleus neurons, usually at the level of the attachment of the rostral medullary velum or in the same plane as the origin of CN III, were obtained from one acute, two subacute, one chronic grass sickness and one control case. Most of the horses were different individuals than those used for the previous study, but all the grass sickness cases had chromatolytic trigeminal ganglia neurons on histological examination.

Table 5-4: Subjects for trigeminal ganglion and mesencephalic trigeminal nucleus CGRP immunohistochemistry studies

Horse ID	Diagnosis	Duration (days)	Samples
98/374	AGS	2	TG
98/417	AGS	6	TG
98/443	AGS	3	Mesencephalon
97/428	AGS	2	Mesencephalon
95/425	SGS	3	TG, Mesencephalon
95/582	SGS	3	TG
96/291	SGS	6	TG
98/563	SGS	3	TG
96/475	CGS	19	TG
97/313	CGS	11	Mesencephalon
97/348	CGS	15	TG, Mesencephalon
97/155	Colic	N/A	TG
97/263	Colic	N/A	TG
97/618	Colic	N/A	Mesencephalon
97/654	Colic	N/A	TG
98/204	Orthopaedic	N/A	TG
98/210	Orthopaedic	N/A	TG
98/387	GME	N/A	TG

(ii) Laboratory methods

Indirect immunoperoxidase staining was performed using a two step indirect immunoperoxidase technique and the MicroProbe system (Fisher Scientific).Tissues were collected and processed to paraffin blocks as previously described. Five μm sections, placed on electrostatically charged slides (ProbeOn Plus, FisherBiotech), were dewaxed in xylene and a graded series of decreasing percentage alcohols and transferred from 70% ethanol to 0.5% H_2O_2 in methanol for 10 minutes to block endogenous peroxidase. Sections were incubated in 10% normal goat serum (Vector Laboratories) for 30 minutes at room temperature in a humid chamber before being incubated with polyclonal rabbit anti synthetic rat CGRP (Serotec Ltd) for 1 hour at 37°C. One section from each tissue was incubated with polyclonal universal negative control serum (DPC Llanberis) was included in each run as a negative control. 1:100 Goat anti Rabbit immunoglobulin conjugated to HRP (Sigma Immuno Chemicals) was applied as the secondary antibody for 30 minutes at room temperature in a humid chamber. Sections were developed by using a DAB substrate kit (Vector Laboratories) at room temperature for ten minutes and counterstained with hematoxylin. Finally, the slides were dehydrated in graded alcohols and three changes of xylene and mounted in Permount (Fisher Scientific).

(iii) Quantitative Methods

Sections were magnified to x100 and captured, outlined and the diameter measured by image analysis software as for the trigeminal ganglion morphometry study (page 162). A variation on systematic random sampling of squares (Mayhew and Sharma, 1984) was used to obtain a systematic random pattern of fields covering each montage. The first field was chosen at one extreme of the section and all neurons not touching the side of the field were outlined. Between six and nine fields were chosen to cover the section until at least 100 cells had been outlined. In order to avoid measuring fields that were not stained adequately, only fields in which there was at least one positive immunoreactive cell were included. Individual cells were divided into positive or negative categories and outlined separately: In order to prevent the inclusion of artefactually stained cells, only cells that appeared to have positive

staining throughout the cell were included as 'positive'. Similarly, cells were labelled as 'negative' only if they did not show any positive staining. A few cells which did not match these criteria were not included. Measurement data was obtained for labelled and unlabelled neurons in control and grass sickness cases and analysed using statistical software (Excell, Microsoft Inc and C-Stat, Oxford Scientific).

Having outlined the neurons, captured fields were reloaded and the proportion of positive staining cells which stained intensely positive was assessed. Intensely staining cells were categorised separately as 'strong positive' cells. In order to add an element of objectivity, the degree of immunoreactivity was defined as a range of colour intensities using the image analysis system. However, since the background staining and light intensity differed from section to section, a degree of operator assessment was still required.

c) Results

CGRP immunoreactive cells (CGRP-IR) in trigeminal ganglia exhibited a granular diffuse cytoplasmic brown staining, with a range of staining intensities (Figure 5-1). No convincing CGRP expression was seen in any mesencephalic nucleus of V neurons but in very few neurons pale, patchy staining was seen associated with the soma. Careful observation of a number of trigeminal ganglia sections suggested that the CGRP-IR cells were in many cases smaller than unlabelled cells. It also appeared that EGS tissues contained fewer strongly labelled neurons than control tissues (Figure 5-2 and Figure 5-3). The results from quantitation of many sections for each case are presented in Table 5-5.

The majority of summed EGS and control diameter data for labelled and unlabelled neurons had a normal appearing distribution but failed the equal variance test. Statistical tests (ANOVA on ranks, Dunn's multiple comparison procedure, $p<0.01$) confirmed that positive staining neurons were smaller than unlabelled neurons both in EGS and control tissues, and that the diameter data for EGS and control unlabelled neurons were not significantly different from each other. CGRP-IR neurons were

generally larger in EGS than in control tissues. EGS cases had a larger proportion of positively labelled neurons but a much smaller proportion of strongly labelled cells than did control cases (Table 5-6).

Diameter data for unlabelled cells and EGS and control labelled neurons were compared to diameter data obtained for normal and chromatolytic TG neurons from EGS cases in the previous section. All the groups were significantly different from each other (ANOVA on ranks, $p<0.001$, Dunn's multiple comparison procedure).

Table 5-5: TG CGRP individual subject immunoreactivity and diameter morphometry

Horse	Diagnosis	Number Negative	Mean (SD) Negative	Number Positive	Mean (SD) Positive	Prop Str pos
97/155	Control	30	38.3 (8.7)	67	37 (7.6)	0.66
97/263	Control	49	41.6 (8.7)	63	35 (6.4)	0.17
97/654	Control	53	47.0 (13.8)	89	23 (11.7)	0.85
98/204	Control	76	47.5 (11.2)	79	331 (6.6)	0.73
98/210	Control	57	44.1 (10.6)	61	34 (5.0)	0.66
98/387	Control	45	52.6 (9.9)	56	37 (6.1)	0.55
98/374	AGS	44	48.3 (13.7)	61	34 (6.8)	0.61
98 417	AGS	65	41.3 (12.7)	43	36 (6.2)	0.12
95/425	SGS	47	44.1 (11.2)	65	35 (8.6)	0.15
95/582	SGS	48	47.6 (12.8)	54	32 (8.1)	0.09
96/291	SGS	33	49.0 (13.4)	75	39 (8.3)	0.04
98/563	SGS	47	50.8 (12.6)	62	42 (11.5)	0.05
96/475	CGS	51	45.2 (11.6)	48	38 (7.5)	0.21
97/348	CGS	73	49.0 (13.6)	34	38 (9.4)	0.26

N = Number included
Negative = no staining
Positive = immunoreactivity
Prop Str pos = proportion of CGRP-IR cells showing strong immunoreactivity
Mean diameter measurements in micrometers. Standard deviations are given in perentheses.

Table 5-6: TG neuron CGRP EGS and control subject summary data

	Mean (SD)		Percentage of neurons		
	Negative	Positive	Neg	**Pos**	Str pos
EGS	47 (12.8)	37 (8.8)	19	81	13%
Control	46 (11.5)	32 (9.3)	43	57	64%

Str. pos = strongly positive staining neurons given as a proportion of positive neurons

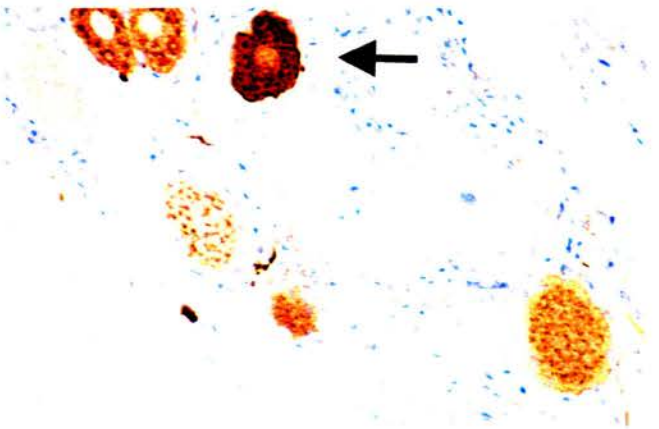


Figure 5-1: Control trigeminal ganglion, anti CGRP immunohistochemistry showing the variation in cellular staining. One cell is a strong positive (arrow). x 400.

Figure 5-2: Control Trigeminal ganglion. x100.

Figure 5-3 :Trigeminal ganglion, Chronic Grass Sickness. x 100

d) Discussion

Morphometric data from all groups examined indicates that CGRP is expressed preferentially by smaller neurons in the equine trigeminal ganglion. This agrees with data from laboratory animals in which CGRP is expressed throughout the trigeminal ganglion but is confined to neurons smaller than 50 μm, with the majority of cells labelled in pigs and cats being smaller than 30 μm in diameter (Lazarov, 1995; Aigner *et al*, 1997). These published morphometric results are similar to the 32 μm mean diameter of CGRP-IR trigeminal ganglion neurons observed here in control horses, and places equine CGRP-IR neurons into the category of type B neurons [mean diameter in pigs = 20 –50 μm, Aigner, 1997 #576]. It is likely that strong positive equine CGRP-IR trigeminal neurons (mean diameter 28 μm) would fall into the type C category (mean diameter <20 μm) if it were adjusted for the larger size of equine neurons. In order for the results to be comparable to the TG morphometry study, cells were outlined regardless of whether or not they contained a nucleus. This could have resulted in bias towards small positive cells since these are easier to identify than small negatively stained cells. Being aware of this however, a concerted effort was made to include negative cells equally.

CGRP has been of considerable interest in providing a specific and prevalent marker for type B sensory ganglion neurons (Silverman and Kruger, 1989). Studies in laboratory animals focusing on CGRP-IR in dorsal root ganglia have concluded that CGRP-IR somata are most frequently associated with high-threshold mechanosensitive, presumably nociceptive, afferent neurons. CGRP however can be expressed not only in nociceptive but also in many other types of primary afferent neurons, the condition being that the conduction velocity is slow , <2.5 m/s, and/or the cell soma small, <40μm diameter (Hoheisel *et al*, 1994). However, there is no neuropeptide marker known at present that can be considered typical for a particular type of afferent unit [Hoheisel, 1994 #581, or can be correlated in any exclusive manner with any pathological states (Cooper *et al*, 1996).

It is interesting that this study, and others, have been unable to find CGRP-IR neurons in the mesencephalic trigeminal nucleus (Lazarov, 1995; Lazarov and Chouchkov, 1996). MTN neurons are singled out by being the only primary afferent neurons in CNS and as such might be expected to express CGRP. MTN neurons are thought to process proprioceptive information from masticatory and extrinsic eye muscles and periodontal ligament fibres but very little is known about their neurochemical organisation (Lazarov, 1995). The nucleus is composed of large and medium sized pseudounipolar primary afferent neurons as well as many smaller multipolar MTN neurons. In studies using immunocytochemical retrograde tracing techniques, immunoreactivity could not be detected in cell bodies against any ten neuropeptides, including CGRP (Lazarov and Chouchkov, 1996).

Unlike classical neurotransmitters which are synthesised at the synapse, neuropeptide synthesis is limited to the cell soma, from where it is transported down the axon (Cooper *et al*, 1996). Thus, despite the concentrations of any given peptide in the brain being maximally two to three orders of magnitude lower than classical neurotransmitters (Cooper *et al*, 1996), CGRP producing neurons should be immunoreactive at the level of the soma. The very occasional association of mesencephalic V neurons with pale CGRP immunoreactivity in this study may be explained by the electron microscopic observation that CGRP expressing perisomatic fibres in the mesencephalon, probably derived principally from the brainstem reticular formation and the spinal trigeminal nucleus, can be in direct apposition to perikarya of unstained large cells (Lazarov and Chouchkov, 1996). Some of these CGRP expressing fibres make synaptic contacts with mesencephalic V cell bodies and dendrites. Since the faint staining was seen throughout the soma however, it is possible that very few equine MTN neurons, and therefore probably also laboratory animal MTN neurons, do express CGRP.

Although there was a great deal of individual variation, TG ganglia from control horses had significantly fewer CGRP-IR neurons but a greater proportion of strongly immunoreactive neurons than did EGS cases. Furthermore, CGRP-IR neurons which were present in EGS ganglia ($p<0.0001$) were significantly larger than in control

ganglia while there was no significant difference in the size of unstained EGS and control TG neurons (p=0.24). Neuropeptides have long been considered to act as neurotransmitters or neuromodulators but in addition they may potentiate the action of acetylcholine by increasing the number of receptors on the postsynaptic membrane (Thompson *et al*, 1995). In this way they may contribute to a variety of regulatory and trophic neuronal functions (Lazarov, 1995). CGRP levels in motor neurons increase after axotomy (Wang *et al*, 1993b; Thompson *et al*, 1995), suggesting a role for this peptide in the response of neurons to injury and in regeneration. Based on the available data one can formulate the hypothesis that CGRP expression by additional, larger diameter, trigeminal neurons in Equine Grass Sickness is due to a similar mechanism, and that a smaller proportion of EGS neurons are strongly immunoreactive as a number of them have become chromatolytic.

The observation that the diameters of normal trigeminal neurons are larger than chromatolytic neurons, and that small EGS neurons are more likely to be chromatolytic may suggest that CGRP expressing neurons are preferentially targeted in EGS. This is supported by the observation that mesencephalic V neurons neither express CGRP nor are chromatolytic in EGS cases. It is tempting to correlate the decrease in CGRP expression with clinical signs shown by EGS cases and a prominent candidate is rhinitis sicca, which is so commonly seen in chronic EGS cases. CGRP often co-localises with substance P and both substance P and CGRP have vasodilator functions, it has been known for many years that antidromic stimulation of afferents produces a vasodilator response (Cooper *et al*, 1996), increases capillary permeability and augments the secretion of submucous glands (Zhao and Tao, 1994). This efferent role of some of these peptidergic afferent axons may also be inferred from their specific distributions. Sites involved in regulating access to and sensitivity of sense organs to external stimuli (e.g. cochlear and vestibular hair cells, taste bud orifices, and main olfactory epithelium) are heavily innervated by peptidergic neurons and may serve versatile roles in response to noxious chemical stimuli, permitting sensory axon modulation of autonomic neuron activity and triggering of protective reflexes (Silverman and Kruger, 1989).

Dysfunction of any of these at the level of the nasal mucosa, but particularly a decrease in secretion of submucous glands, is a very reasonable pathogenesis for an alteration in the mucosa resulting in rhinitis sicca.

CGRP is also known to have an impact on gastric motility, and the response of spinal sensory neuron terminals in the celiac and cranial mesenteric ganglia to noxious stimuli are believed to partly mediate postoperative ileus. CGRP is generally associated with inhibition of gastric motility, but a chronic alteration in these extraspinal intestinogastric inhibitory reflexes could reasonably lead to a paradoxical decrease in gastric motility, such as may be seen in EGS cases. Initial work detailing CGRP changes in the gastrointestinal tract of EGS cases is supportive of this hypothesis (Kitamura, Obihiro University, Japan, work in progress).

In conclusion, CGRP-IR neurons in the equine TG ganglion are smaller than unreactive cells, and chromatolytic neurons in EGS are smaller than normal appearing neurons. This is the first study to correlate neuronal susceptibility to the agent responsible for EGS with a neuronal marker. It suggests that CGRP expressing neurons may be specifically targeted in EGS, and that dysfunction of CGRP expressing neurons may be implicated in the pathogenesis of rhinitis sicca and other clinical signs.

Chapter Six

FINAL DISCUSSION

This work achieved the objective of the study and quantified the histopathology of the CNS of equine grass sickness cases, initiated the investigation of the CNS cytopathology and evaluated the neuroanatomic basis of selected clinical signs.

Equine Grass Sickness cases ultimately are euthanised because of dysfunction of the gastrointestinal system, and research efforts have understandably focused on the autonomic nervous system. The recognition that EGS is a multiple system disease with a highly specific somatic component however, may direct the emphasis towards finding an aetiologic agent selective for neurons in the GVE, GSE and GSA systems. The distribution of CNS lesions is so specific that there can be little doubt that domestic carnivores with dysautonomia share a common aetiologic process, further narrowing the field of possible causes. Indeed it is reasonable to expect any species affected with this disease to have similar CNS changes, and thus it is very likely that hares (personal observation) are affected but the diagnosis in one llama (Kik and van der Haage, 1999), which was reported to have none of the appropriate central changes, may be in doubt.

The results of this study agree with Barlow's observation (1969) that central pathology may be a specific part of the pathology of grass sickness but disagree with the conclusion that the extent is in direct proportion to the duration of clinical illness and is not a primary feature of the disease. CNS pathology was found in 90% of AGS cases in this study, many of which had only been showing clinical signs for a matter of hours. This strongly suggests that Gilmour's hypothesis (1973) is correct and damage to central tissues occurs coincidentally with alimentary pathology.

The suggestion that the causative agent of EGS may reach neurons by retrograde axonal transport (Griffiths *et al*, 1994) should be questioned in light of these results. Although it is probable that the onset of cytopathology greatly precedes the expression of clinical signs, and that a retrograde axonal transport rate for toxins of ~180 mm/day (Stockel *et al.* 1975) may allow central pathology to occur at the same time as the onset of enteric clinical signs, it is unlikely that the resulting pathology could be that neuroanatomically

specific. The route of exposure of the axons of CN III, IV and VI to the agent is particularly difficult to imagine.

The study of equine cytopathology is greatly impeded by the lack of specific reagents or positive control tissues for that species. It was hoped that antigens well conserved throughout evolution, such as ubiquitin, would be recognised by rodent specific antibodies, but the lack of known positive staining equine CNS tissues made it extremely difficult to validate the histochemical techniques. Staining for further heat stress proteins such as alpha B crystallin, detected in chromatolytic neurons in human neurodegenerative diseases (Lowe *et al*, 1992), was attempted repeatedly but the results could not be interpreted despite the use of equine eye lens tissue on positive control sections. The procedures reported in this study all had specific staining in positive control tissues from other species, and no staining in negative control tissues in which the histochemical agents or antibodies had not been applied.

The observation that smaller dorsal root ganglion neurons expressing CGRP become chromatolytic in EGS may indicate that neurons expressing that neuropeptide are particularly sensitive to the pathogenic agent. This is supported by the fact that CGRP is specifically present in the somatic efferent and afferent neurons (Grunditz *et al*, 1994; Thompson *et al*, 1995; Cooper *et al*, 1996) which become chromatolytic in EGS. It is reasonable to suppose however that CGRP expression is only acting as a marker for a specific subset of neurons, and further work allowing the classification of equine neurons into categories such as those described by Aigner (1997) may be profitable. A number of neurochemical substances including substance P, somatostatin, fluoride resistant acid phosphatase, 5-hydroxytryptamine, glutamate and aspartate (Kai-Kai, 1989; Tracey *et al*, 1991) are also localised to small and intermediate diameter neurons in rodent ganglia and may indicate that a factor such as metabolic activity makes neurons susceptible to EGS.

CGRP expression was investigated in central GSE LMNs and was also found to be decreased in chromatolytic EGS neurons. On reflection however the specificity of a

change in neuropeptide expression in chromatolytic neurons was to be expected. Nissl granules are made of rough endoplasmic reticulum and free polyribosomes, and are the site of synthesis of cytoskeletal elements and proteins such as the neuropeptide neurotransmitters containing signal sequences (Kleinsmith and Kish, 1995; Waggie *et al*, 1999). Chromatolysis is associated with a dispersal in Nissl substance (Bodian and Mellors, 1945) and an increase in RNA content of the nucleus (Thomas *et al*, 1993) allowing free polyribosomes to produce proteins necessary for neuronal repair such as regrowth of an axon (Aldskogius, 1978). Since the production of neuropeptide neurotransmitters requires the ribosome to adhere to the endoplasmic reticulum membrane, and are arguably a non-essential product for an injured cell, it would be reasonable to expect CGRP expression, along with many other proteins, to be down-regulated. A more direct approach to try to determine whether chromatolytic neurons are CGRP expressing cells under normal circumstances would be to evaluate the level of CGRP RNA expression using *in situ* RT-PCR techniques (Kleinsmith and Kish, 1995).

The use of the TUNEL technique did not reveal any evidence of central neuronal cell death regardless of whether or not DNA laddering is taken to be a specific marker for apoptosis. This was further supported by the fact that there was no evidence of fibre type grouping in a muscle innervated by CN VII, or a functional deficit of CN V or VII, or pathology to CN III axons. Additionally, the brain of one horse presented for euthanasia several years after recovering from EGS (Doxey *et al*, 2000) was carefully examined and had no central evidence of cell loss or glial scarring (unpublished observations). This conclusion differs from observations made by Pollin and Griffiths (1987) and encourages further scrutiny of central tissues in species with dysautonomia similar to EGS.

In direct disagreement to the above was finding a nucleus by electron microscopy which appeared to be breaking into apoptotic bodies. Neuronal DNA fragmentation has been reported in both the canine and human brains in normal ageing [Borras, 2000 #795], and finding one apoptotic neuron may not be a reflection of the disease process *per se*, particularly in the absence of any further evidence of central neuronal cell death. Specific and powerful new markers for apoptotic neurons such as endogenous caspase-3

breakdown products (Natha *et al*, 2000) could be applied in the future. However their use has the same limitations encountered in the TUNEL procedure, namely the short time interval available to find cells in the process of undergoing apoptosis and the relatively few large equine LMNs with nuclei present on each tissue section.

The observation that chromatolytic and normal appearing neuronal soma contain phosphorylated neurofilaments suggests that abnormal axonal transport is a feature of central EGS pathology. Labelled neurons with normal appearing Nissl substance may either be recovering, or could be accumulating phosphorylated neurofilaments prior to chromatolysis being evident. Reduced transport of selective cargoes of slow transport, especially tubulin, can be detected months before neurodegeneration in ALS, and represents the earliest detectable abnormality (Williamson and Cleveland, 1999). The recognition that EGS pathology includes a defect in axonal transport demands further work to investigate whether the impairment occurs in neurons other than the LMNs investigated in this study, as well as trying to determine the nature of the deficit.

The loss of a recognizable Golgi structure noted by Griffiths (1993) is a feature which should be examined in CNS neurons. Lectin histochemical identification of Golgi apparatus in central EGS tissues was attempted on several occasions with different batches of agents and with expert assistance, but was discontinued when the technique did not result in consistent labelling of equine control tissues. Neurons with dispersed Nissl substance examined by electron microscopy did not have an appreciable Golgi apparatus, however the preservation was compromised to the extent that comparable organelles in normal appearing neurons were also not clearly defined. It is unlikely that the required tissues can be harvested in less time than accomplished in this study, and the process of aortic transfusion in the horse is challenging. Golgi apparatus specific antibodies are available for use in other species (Gonatas *et al*, 1992), and it would be worth establishing whether the Golgi apparatus epitopes are conserved enough for the antibodies to label the organelle in equine tissues.

The absence of inclusion bodies evident in affected neurons by electron microscopy, answers the question of whether EMND and EGS are extreme expressions of one disease, posed by the workshop on equine neurodegenerative diseases (Hahn *et al*, 1997). Chromatolytic LMNs in EMND are characterised by inclusions composed of aggregated vesicular residues of membranous organelle degradation and marginated derivatives of endoplasmic reticulum (Cummings *et al*, 1993), and there can be little question that this places EGS into a separate category. Similar findings to those in EGS in the central neurons of cats (Pollin and Griffiths, 1987) however, provides further evidence that a common aetiologic agent should be sought.

These initial cytopathological findings are the basis for further work, not only to narrow the field of potential aetiologic agents but also to act as a guide for the changes which can be expected to be found in cultured equine neurons (N. Hudson, R(D)SVS, work in progress) exposed to the appropriate agent in vitro.

The determination of the specific appearance of ptosis in the horse secondary to different insults is relevant in the evaluation of clinical cases. The marked reduction of ptosis secondary to sympathetic denervation with the application of a topical alpha adrenergic agonist, should enable the anatomic location of the lesion to be more accurately determined. In addition, the pathway of the postganglionic sympathetic fibres to the eyelid in the horse is incompletely understood and the ability to recognise sympathetic dysfunction secondary to specific insults may provide functional anatomic data. The technique may also prove useful in the diagnosis of botulism, a disease marked by a muscle paresis secondary to a functional denervation.

The use of alpha adrenergic eyedrops as a simple adjunct diagnostic test for EGS is proving to be a useful technique when applied appropriately. Further data must be collected from stressed and exhausted patients and those in pain but not suffering from EGS to determine the specificity, but clinicians at the R(D)SVS are now routinely applying phenylephrine to suspect cases and to date the test has proved to be very sensitive. Its use in very early or even subclinical cases remains to be investigated, but

interestingly a marked positive response was obtained from one animal which had suffered from EGS several years previously, suggesting that smooth muscle tone of the eyelid, and thus the innervation, had never returned to normal.

Postscript

I used to think I knew, I knew ;
But now I must confess :
The more I know, I know, I know,
I know, I know the less.

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Proceedings of 1st International Workshop on grass sickness, EMND and related disorders (1997) IV

Editorial Prologue

The impetus behind this workshop was the recent recognition in Switzerland of clinical cases of equine grass sickness (EGS) and equine motor neuron disease (EMND) that appeared to display a spectrum of neuronal pathologies. Some horses presented with pure enteric and brainstem lesions (classic EGS pathology), others showed only the typical somatic lower motor neuron changes (classic EMND pathology), while a few 'combination cases' appeared to have the clinical signs and pathological changes consistent with both diseases. Interest in human neurology has been raised by what have been termed 'overlap' syndromes of distinct neurodegenerative diseases and it was speculated that these equine diseases, traditionally separated by clinical signs and geographic location, may be similarly related.

Equine grass sickness was recently demonstrated to be very similar or identical to the disease 'mal seco', known in Patagonia for many decades. The recognition was long overdue and opened up potential new avenues of investigation. The presence of EGS in two such very different environments as Europe and Patagonia and its conspicuous absence from the USA may well hold a key as to its cause.

Some of the clinical as well as the light microscopic neuronal lesions of EGS appear identical to those of EMND despite the difference in the principle target populations of neurons affected. The diametrically different correlation of disease incidence and access to grass in these two diseases also merits further investigation particularly in the light of evidence of oxidative injury being a component in EMND.

Could it be that EGS and EMND represent opposite extremes of a more generalised neurodegenerative process in effect in the Swiss cases? Ultrastructural comparisons between affected somatic neurons in EMND and autonomic neurons from EGS cases may yield further evidence of this process.

Horses with EMND have been observed to have lower serum alpha-tocopherol concentrations than on-farm control horses raising the possibility of a connection to equine degenerative myeloencephalopathy (EDM), occurring usually in much younger equids. Histopathological findings such as the florid neuraxonal dystrophy associated with EDM as well as the widespread lipopigment accumulation found in both EMND and EDM, may be related to the age at which an antioxidant-deficient state was initiated; animals destined to develop EDM having subnormal circulating alpha-tocopherol concentrations at a much younger age than that of the EMND cases examined.

The striking clinical resemblance of EMND to the sporadic form of amyotrophic lateral sclerosis (ALS) in humans and the absence of a suitable spontaneous syndrome in other species opens up the potential that this disease could serve as a model for the human disorder. This should encourage the search for a common trigger in both diseases and the pursuit of lines of investigation fruitful in ALS, such as the potential role of excitatory neurotransmitters, antioxidants and enteroviruses.

The existence of pathological changes compatible with EGS and EMND in the same animal, the realisation that EGS and mal seco are probably the same disease and the evidence that antioxidants play a role not only in EDM but also in EMND blur accepted boundaries of disease identification. The purpose of the workshop was to assemble an international panel of experts to compare the clinical features, neuropathology, epidemiology and pathogenesis of these fascinating equine disorders. This refereed supplement is a synopsis of the presentations and subsequent discussions at the workshop.

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97/424	SGS	3	5	Highland	Mare
98/303	SGS	3	6	Cob	Gelding
98/401	SGS	5	9	Arab x	Mare
98/563	SGS	3	8	Highland	Mare
95/359	CGS	14	8	Coloured	Gelding
96/475	CGS	10	18	Pony	Gelding
97/150	CGS	76	7	Thoroughbred x	Mare
97/293	CGS	21	2	Highland	Mare
97/313	CGS	11	4	Thoroughbred x	Mare
97/348	CGS	15	5	Welsh x	Gelding
97/376	CGS	18	4	Clydesdale	Mare
97/538	CGS	10	4	Thoroughbred x	Mare
97/605	CGS	17	3	Welsh pony	Gelding
98/179	CGS	35	4	Connemara	Mare
98/244	CGS	21	7	Highland	Mare
98/264	CGS	16	4	Thoroughbred x	Gelding
98/502	CGS	26	5	Connemara x	Gelding
98/62	CGS	7	1	Anglo Arab	Mare
476/98	Open	1		Thoroughbred	Gelding
97/1011	Orthopaedic	8		Thoroughbred	Gelding
97/1116	Respiratory	14		Thoroughbred	Gelding
97/352	Orthopaedic	1		Welsh cob	Mare
97/618	Colic	7		Clydesdale	Mare
98/204	Orthopaedic	2		Highland	Mare
98/210	Orthopaedic	7		Thoroughbred	Mare
98/251	Respiratory	6		Thoroughbred	Gelding
98/261	Orthopaedic	5		Irish Draft	Gelding
98/295	Orthopaedic	8		Thoroughbred	Gelding
98/365	Colic	6		Irish Draft	Mare
98/418	Colic	6		Thoroughbred	Mare

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Guest Editorial

Equine Neurodegenerative Diseases—Stressed Neurons and other Radical Ideas

It is almost impossible these days to avoid the subject of 'oxidative stress' when reading about the aetiology, pathogenesis or treatment of acute and chronic neurodegenerative diseases, particularly in human medicine. This emerging field clearly demonstrates to neophytes why 'chemistry' has always been a prerequisite for entry into veterinary school and serves to remind us of the staggering complexity of metabolic interactions required for life.

The term 'degenerative diseases' implies that there is no clear knowledge of the cause and pathogenesis of the diseases in question. These diseases tend to be symmetrical and involve degeneration of selective groups of neurons that may be functionally or anatomically related. Typically, the pathological process is one of slow involution of cell bodies without any intense inflammatory reaction. Oxidative damage is now thought to be a potential contributor to the pathogenesis of these illnesses with substantial evidence accumulating in Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (human motor neurone disease). The paper by de la Rúa-Domènech *et al.* in this issue of the *Journal* associates a prominent equine neurodegenerative disease, equine motor neuron disease (EMND), with the dietary deficiency of an important antioxidant, α-tocopherol.

All aerobic organisms are continually exposed to oxidative stress. The use of oxygen as a final electron acceptor produces more energy per molecule of glucose, but carries a high cost due to the concomitant production of reactive oxygen species (ROS). These ROS include non-radical oxygen derivatives such as hydrogen peroxide (H_2O_2) in addition to the oxygen-centred free radicals such as superoxide radical ($O_2\cdot^-$), nitric oxide (NO·) and the highly reactive hydroxyl radical (OH·). A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons. In an effort to complete its own electron orbitals by extracting an electron from a neighbouring molecule however, a further free radical can be generated, potentially leading to a chain reaction. Transition metals such as iron and copper, whose release is facilitated by trauma and other forms of cytotoxicity, promote these reactions due to their loosely bound electrons. The extent of damage done by oxidative stress is considerable, including derangements of cell metabolism, DNA breakage, protease and nuclease activation and peroxidation of lipids.

Neurons appear to be at particular risk of free radical damage by mechanisms that are not entirely understood. Neurons are deficient in some protective components such as catalase, glutathione peroxidase and vitamin E while being subjected to free radical attack, including that produced by the product of oxidation of the neurotransmitter dopamine and by the induction of NO synthase after peripheral nerve injury and chronic central nervous system (CNS) inflammation. Motor neurons are particularly susceptible by having high energy requirements associated with the maintenance of long axons and by the additional fluxes of NO induced when the brain adapts to motor neuron injury by rearranging synapses with other neurons. In addition, the high concentration of polyunsaturated fatty acids, in neuronal cell membranes makes these cells particularly prone to lipid peroxidation. This process is accelerated by transition metals, such as iron, that accumulates in the globus pallidus and substantia nigra (containing target neurons in Alzheimer's disease), as well as being present in CSF.

Luckily, several antioxidant defence mechanisms are in place, including the segregation of the majority of oxidation–reduction reactions occurring within mitochondria, the binding of tran-

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sition metals to proteins, and the existence of chain-breaking antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase. Finally, and of importance in the context of EMND, the active component of vitamin E, α-tocopherol, is the major lipid soluble antioxidant and has a high reactivity with organic peroxyl radicals derived from lipid oxidation.

In reality, things are rather more complicated of course, and the obvious solution of removing transition metals and adding antioxidants is unlikely to be the answer to oxidative stress-induced diseases. Many ROS have important biological functions, the messenger roles of nitric oxide being one example. Metal ion chelators similarly are not useful in the long-term due to the important physiological function of iron and because the chelates themselves are able to catalyse free radical reactions, a problem shared by most antioxidants ('radicals beget radicals'). Furthermore, hydroxyl radical scavengers, such as mannitol, ethanol and dimethyl sulphoxide, react by diffusion-controlled rates. So for a molecule to scavenge OH· *in vivo* it must be present at higher concentrations than surrounding biological molecules. This probably (unfortunately) means that for ethanol to do one any good, one has to get well and truly 'pickled'! It is possible that increasing SOD activity is actually damaging, due to the hydrogen peroxide produced during dismutation of superoxide, and the therapeutic use of antioxidants such as SOD and tocopherols have been disappointing.

Increased ROS formation has been implicated in many human diseases but has not yet been *proven* to play the major pathologic role in any of them. As de la Rúa-Domènech *et al.* point out, it is unclear whether the decreased serum α-tocopherol concentration and oxidative injury have a causative role in EMND or are epiphenomena or consequences of the disease. To prove that ROS species are important in a particular disease it has to be shown that ROS are formed at the site of primary injury, that removal of ROS or prevention of their formation has beneficial effects and that direct application of ROS at concentrations found *in vivo* reproduces all or most of the primary injury. This is going to be one of the challenges in the pursuit of the aetiology of equine neurodegenerative diseases.

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